12-1-2015

UNDERSTANDING CHYTRIDIOMYCOSIS RESISTANCE BY INVESTIGATING THE CUTANEOUS DEFENSE MECHANISMS OF MARSUPIAL FROGS

David Burkart
Southern Illinois University Carbondale, davidburkart@siu.edu

UNDERSTANDING CHYTRIDIOMYCOSIS RESISTANCE BY INVESTIGATING THE CUTANEOUS DEFENSE MECHANISMS OF MARSUPIAL FROGS

by

David Burkart

B.S., N.C. State University, 2007

A Thesis
Submitted in Partial Fulfillment of the Requirements for the
Master of Science Degree

Department of Zoology
in the Graduate School
Southern Illinois University Carbondale
December 2015

THESIS APPROVAL

UNDERSTANDING CHYTRIDIOMYCOSIS RESISTANCE BY INVESTIGATING THE CUTANEOUS DEFENSE MECHANISMS OF MARSUPIAL FROGS

By

DAVID BURKART

A Thesis Submitted in Partial

Fulfillment of the Requirements

for the Degree of

Master of Science

in the field of Zoology

Approved by:

Dr. Alessandro Catenazzi, Chair

Dr. Vance Vredenburg

Dr. Robin Warne

Dr. Laurie Achenbach

Graduate School
Southern Illinois University Carbondale
November 6th, 2015

AN ABSTRACT OF THE THESIS OF

David Burkart, for the Master of Science degree in Zoology, presented on the 6th of November, 2015, at Southern Illinois University Carbondale.

TITLE: UNDERSTANDING CHYTRIDIOMYCOSIS RESISTANCE BY INVESTIGATING THE CUTANEOUS DEFENSE MECHANISMS OF MARSUPIAL FROGS

MAJOR PROFESSOR: Dr. Alessandro Catenazzi

Anurans are declining worldwide because of the spread of *Batachochytrium dendrobatidis* (Bd), the fungus that causes chytridiomycosis. However, some frogs are resistant to this disease, and understanding why may be critical to saving those that are susceptible. In Peru, *Gastrotheca excubitor* is resistant to chytridiomycosis while *Gastrotheca nebulanastes* is susceptible. Two anuran skin defenses, symbiotic bacteria and antimicrobial peptides (AMPs), have demonstrated the ability to inhibit Bd *in vitro* when isolated from certain frogs. We tested if these defenses can explain the difference in susceptibility between the two *Gastrotheca* species. The cutaneous bacteria and AMPs of both species were collected, tested for their abilities to inhibit the growth of Bd, and analyzed for their compositions. Results indicate that 34%of the strains of skin bacteria from *G. excubitor* were able to inhibit the growth of Bd whereas only 10% isolated from *G. nebulanastes* were effective. *Gastrotheca excubitor* also has stronger anti-Bd skin bacteria. Neither frog species has peptide mixtures capable of completely inhibiting Bd, and overall species did not differ in the anti-Bd abilities of their peptides. These results suggest that the chytridiomycosis resistance experienced by *G. excubitor* may be attributed to its skin bacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER 1

NATURAL ANURAN DEFENSES AGAINST CHYTRIDIOMYCOSIS: THE ROLES OF ANTIMICROBIAL PEPTIDES AND CUTANEOUS BACTERIA

INTRODUCTION:

Amphibians are currently one of the most endangered vertebrate groups. Approximately one third of all amphibian species are listed as threatened by the IUCN, and in the past several decades there have been over 100 species extinctions and thousands of populations have experienced significant declines (Stuart et al., 2004). Many factors are contributing to amphibian declines including habitat loss, climate change, and the spread of infectious diseases (Fisher and Shaffer, 1996, Davidson et al., 2001, Marsh and Trenham, 2001, Pounds et al., 1997, Pounds et al., 1999, Kiesecker et al., 2001, Carey and Alexander, 2003, Daszak et al., 2003). One of these infectious diseases, chytridiomycosis, may be the most significant contributor to amphibian declines.

Chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (Bd). It is an emerging infectious disease that has been linked to the extinctions and extirpations of numerous amphibian species world-wide (Daszak and Berger, 1999, Berger et al., 1998b, Skerratt et al., 2007, Fisher et al., 2009). Bd was first described when the fungus was isolated from dead and dying frogs from Australia and Central America (Berger et al. 1998). Effective treatment strategies for chytridiomycosis are critical because it is unlikely that Bd will be eradicated form the environment. Chytridiomycosis is currently treated by various anti-fungal drugs or by heat therapy.

While both strategies are effective, none of these have any applications for frogs *in situ*. A strategy that can protect the remaining vulnerable frogs may allow for populations to persist in their natural environment.

Because some frog species are resistant to Bd, their natural defenses are being studied for their abilities to inhibit fungal growth. Two of these defenses, antimicrobial peptides (AMPs) and cutaneous skin bacteria, show promise. AMPs and cutaneous bacteria from many frog species have already demonstrated anti-Bd capabilities. Perhaps one or both of these defenses could be applied to or amplified in susceptible frogs as an enriching defense against Bd.

ROLE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN AMPHIBIAN DECLINES:

Bd infects its amphibian host with motile zoospores in aqueous environments. The zoospores infect keratinized mouthparts on tadpoles or keratinized epidermal skin cells on adults. Following infection, the fungus grows as a thallus and eventually produces reproductive structures, the zoosporangia, where new zoospores are produced and released (Berger et al., 2005a, Kilpatrick et al., 2010). Infection causes reduced electrolyte transport, impairing osmoregulation and ultimately causing death by asystolic cardiac arrest (Voyles et al., 2009). The fungus has been shown to spread geographically very quickly (Vredenburg et al., 2010). Once arriving in a new location, Bd can quickly reduce the population size of the naive frog species (Lips et al., 2006). Bd has had a global impact on amphibian populations. To date significant declines attributed to Bd have occurred in Australia, Panama, Costa Rica, Peru, and the United

States (Pounds et al., 1997, Berger et al., 1998b, Lips et al., 2008, Catenazzi et al., 2011, Vredenburg et al., 2010, Vredenburg et al., 2011).

AMPHIBIAN IMMUNE SYSTEM:

To fight off disease, frogs possess both innate and adaptive immune systems, as well as products metabolized by symbiotic skin bacteria (Harris et al., 2006, Harris et al., 2009a, Brucker et al., 2008). The innate immune system is non-specific and acts quickly upon infection by a pathogen. This system includes phagocytic cells, lysozymes, and antimicrobial peptides (AMPs). The adaptive immune system includes antigen-specific T and B lymphocytes that are created by previous exposure to their specified pathogen. The adaptive immune system does play some role in defense against chytridiomycosis as reduced lymphocyte numbers results in increased intensity of infection (Ramsey et al., 2010). However, Bd has been shown to inhibit lymphocyte production and induce apoptosis, which interferes with the development of an effective cell-mediated defense (Fites et al., 2013a, Fites et al., 2013b). Since Bd infects the keratinized epidermal cells of the skin, a frog's cutaneous defenses are the first line of defense against chytridiomycosis (Rollins-Smith et al., 2002b). Therefore, these defenses, which include AMPs and cutaneous bacteria, may be very important in providing protection against chytridiomycosis.

ANTIMICROBIAL PEPTIDES:

AMPs are synthesized in granular glands located on the frog's skin where they are stored as preproproteins (Bovbjerg, 1963, Mills and Prum, 1984, Rollins-Smith et al.,

2005). Activation of the sympathetic nervous system stimulates certain nerve receptors in the muscle tissue surrounding these glands (Benson and Hadley, 1969, Dockray and Hopkins, 1975, Sjöberg and Flock, 1976, Holmes and Balls, 1978), and the preproprotein is cleaved to release an activated AMP (Resnick et al., 1991, Amiche et al., 1999, Bowie et al., 1999). The activated peptide is secreted from the granular gland and combined with mucous secreted from the frog's cutaneous mucous glands, coating the skin (Schumacher et al., 1994, Rollins-Smith et al., 2011, Gammill et al., 2012). Most AMPs are cationic and characterized by hydrophobic regions (Nicolas and Mor, 1995, Simmaco et al., 1998, Zasloff, 2002). They are therefore able to bind to negatively-charged regions on their target's lipid membranes (Nicolas and Mor, 1995) and lyse their target cells by either embedding into the membrane's matrix or by creating disruptive pores that leak intracellular material (Amiche et al., 1999, Conlon et al., 2004b, Pasupuleti et al., 2012).

Early research on frog AMPs focused on refining the methodology of collecting and isolating them and identifying individual peptides (Dockray and Hopkins, 1975, Tyler et al., 1992, Bowie et al., 1999). These isolates were then assayed against pathogens, revealing that many peptides were effective against bacteria, fungi, protozoa, and viruses (Nicolas and Mor, 1995, Simmaco et al., 1998, Conlon et al., 2004a, Apponyi et al., 2004, Zasloff, 2002, Rinaldi, 2002). Research then shifted to a conservation approach in response to amphibian declines. *In vitro* assays were developed to test frog peptides for activity against pathogens like Bd (Rollins-Smith et al., 2002a, Rollins-Smith et al., 2002b, Rollins-Smith et al., 2002c). Most of the peptides used in this early testing

demonstrated anti-Bd activity, including those isolated from a declining frog susceptible to Bd, *Rana tarahuamara* (Rollins-Smith et al., 2002c).

Drastic frog population declines in Australia led to extensive research on the AMPs of Australian frogs. The peptide compositions of many of these frog species are well documented (Bowie et al., 1999), and when assayed against Bd most of these peptides inhibited growth regardless of source host susceptibility, although AMP effectiveness did decrease with host susceptibility (Woodhams et al., 2007a). The AMPs of the declining North American frog *Rana muscosa* have also been assayed for activity against Bd, revealing that these frogs have a rich repertoire of anti-Bd AMPs despite being susceptible (Woodhams et al., 2007b). Therefore, the presence of anti-Bd AMPs does not always correlate with resistance to chytridiomycosis. This relationship has also been demonstrated in other frogs from different families distributed worldwide, including: *Alytes obstetricans* in Europe (Bosch and Rincón, 2008), *Silurana tropicalis* in Africa (Parker et al., 2002), and *Hylomantis lemur* in South America (Conlon et al., 2007b).

CUTANEOUS BACTERIA:

The diverse community of bacteria that naturally occurs on frogs' skins are another important component to their immune systems. These bacteria are probably established on the skin when frogs are young (Fierer et al., 2012). Species that exhibit parental care may transmit bacteria vertically (Walke et al., 2011). Symbionts are also probably transmitted horizontally during mating and hibernating aggregations or acquired from the environment (Muletz et al., 2012). It is suggested that within an amphibian community, cutaneous microbe composition vary more among species than across

space (McKenzie et al., 2012), indicating some sort of genetic regulation of symbiont community structure. This microbial regulation may be controlled by the frog's immune system, such as AMPs (Fierer et al., 2012); however, the establishment and composition of skin bacteria most likely depends on complex interactions among microbes and with the host's mucosome (Scheuring et al., 2012). There may also be correlations between a frog's diet and cutaneous microbe species richness, abundance, and community structure. For example, groups of captive *Agalychnis callidryas* fed diets varying in carotenoid composition/abundance differed in the skin microbes that they harbored (Antwis et al., 2014).

While serving a variety of functions, some of these bacteria contribute to their host's immunity. They may destroy pathogens by excreting metabolites, inhibit pathogen colonization by competition, or enhance other components of the host's innate immune system (Harris et al., 2006, Brucker et al., 2008, Flechas et al., 2012). As with AMPs, cutaneous bacteria are currently being sampled and assayed for their effectiveness against Bd (Harris et al., 2006, Flechas et al., 2012). Cutaneous bacteria have been sampled from the skins of many frog species, cultured, and assayed *in vitro* for their abilities to inhibit the growth of Bd. These assays typically involve growing a fungal "lawn" of Bd across a petri dish and streaking the query bacteria on one side and a negative control bacteria on the other side (Flechas et al., 2012, Brucker et al., 2008, Harris et al., 2009a). Anti-fungal activity is measured by clear zones of inhibition around the query bacteria. However, the design of these assays allow for only semi-quantitative measurements of anti-Bd activity and it is difficult to control for the amount of Bd zoospores tested (Bell et al., 2013). To mitigate these limitations a new co-culture assay

has been developed with a design similar to the AMP-Bd inhibition assay developed by Rollins-Smith (2002a) (Bell et al., 2013).

In the United States, the bacterium *Janthinobacterium lividum* occurs naturally on many native amphibian species and produces the anti-fungal metabolite violacein (Brucker et al., 2008). It has been demonstrated that this metabolite is capable of inhibiting the growth of Bd *in vitro* (Brucker et al., 2008, Harris et al., 2009a). The capabilities of *J. lividum* to protect amphibians from Bd has been studied extensively. This bacterium naturally occurs on the salamander *Plethodon cinereus*, and when these salamanders were experimentally augmented with *J. lividum* they had increased levels of violacein and were more likely to survive experimental infection with Bd (Becker et al., 2009). Additionally, experimentally removing *J. lividum* form the skins of *P. cinereus* decreased an individual's likelihood to survive experimental infection with Bd (Becker and Harris, 2010).

Frogs in the mountain yellow legged frog complex also naturally harbor *J. lividum*. Some of these frogs have been experiencing dramatic population declines due to chytridiomycosis (Briggs et al., 2005, Rachowicz et al., 2006). However, it is suggested that among and within mountain yellow legged frog species, populations with larger quantities of anti-Bd bacteria do not experience declines (Woodhams et al., 2007a, Lam et al., 2010). For example, more individuals from non-declining populations of *Rana sierrae* may harbor more anti-Bd bacteria than individuals in declining populations of *Rana muscosa* (Woodhams et al., 2007a). Additionally, a separate population of *R. muscosa* that was found to be composed of a high number of individuals with anti-Bd bacteria was not in decline (Lam et al., 2010). *J. lividum* was common among the anti-

Bd bacteria found in these studies. This bacterium has been demonstrated to protect susceptible *R. muscosa* from Bd infections *in vivo* because individuals exposed to *J. lividum* prior to infection with Bd survived whereas those that were not inoculated with *J. lividum* did not (Harris et al., 2009a). An emergency inoculation with *J. lividum* of a declining population of *R. muscosa* in the wild was executed, and this population continues to persist despite the presence of Bd (Vredenburg et al., 2011).

AMPS, CUTANEOUS BACTERIA, AND CONSERVATION:

The results accrued from research involving frog AMPs and cutaneous bacteria and their abilities to inhibit the growth of Bd have inspired potential conservation strategies. The most feasible of these strategies may be the application of anti-Bd bacteria as probiotics by bioaugmentation therapy (Bletz et al., 2013). This strategy involves amplifying the presence of naturally occurring beneficial symbionts to better protect the host. Bioaugmentation with the anti-Bd bacterium *J. lividum* has already been successful *in vivo* with both the salamander *P. cinereus* (in captivity) and the frog *R. muscosa* (Harris et al., 2009a, Muletz et al., 2012, Vredenburg et al., 2011).

Perhaps bioaugmentation therapy could also be used to save wild populations of susceptible frogs, but the transition from laboratory experiments to wild populations will come with challenges. There has been little research on applying this strategy in natural systems (Bletz et al., 2013), and abiotic factors will certainly influence the success of a probiotic when applied *in situ*. For example, mineral composition in the soil may influence the efficacy of bioaugmentation for plant crops *in situ* (Haas and Defago, 2005). For frogs, it is unrealistic that every individual in a threatened population could be

collected and treated with a probiotic. Fortunately, vertical and horizontal transfer would help propagate the beneficial bacterium throughout a population (Walke et al., 2011, Muletz et al., 2012). Also, the results from the extensive work done with the mountain yellow legged frogs suggest that herd immunity, which occurs when a pathogen's effects are inhibited by a high proportion of immune hosts, may prevent Bd outbreaks (Woodhams et al., 2007a, Lam et al., 2010). There is evidence that bioaugmentation can occur via environmental vectors such as soil, which gives hope that this strategy could be possible to implement without the need to catch frogs (Muletz et al., 2012).

One important limitation to bioaugmentation is that the bacterium used must naturally occur on the host, and, depending on how it will be applied, in the environment. Failed attempts have occurred when trying to use bacteria from other hosts. For example, *J. lividum* could not be inoculated on the harlequin toad *Atelopus zeteki* (Becker et al., 2011) from Panama, where *J. lividum* has not been reported. Similarly, skin microbes (in the form of whole skin washings) could not be inoculated from the glass frog *Espadarana prosoblepon* onto the rocket frog *Colostethus panamansis* (Küng et al., 2014). Also, some anti-Bd bacteria fail to establish on frogs' skins despite being generously applied (Woodhams et al., 2012b, Küng et al., 2014). It has been proposed that potential probiotics pass rigorous criteria testing before being considered for bioaugmentation (Bletz et al., 2013).

The applications of anti-Bd AMPs towards conservation are less tangible given the current research. In fact, there have been almost no attempts to apply what is known about AMPs and their abilities to inhibit Bd towards protecting live frogs. In an un-successful attempt, anti-Bd AMPs from the resistant frog *Pelophylax esculentens* were

applied to the susceptible toads *Bufo bufo,* but failed to protect toads from Bd infection (Woodhams et al., 2012b).

Anti-Bd AMPs may not be successful when applied as a therapeutic agent, but, if AMP expression is heritable, their presence within a population could be amplified via selective breeding. There are captive reassurance colonies of Panamanian frog species whose natural populations have declined because of Bd. These frogs would be ideal candidates for a selective breeding strategy because the goal of the breeding program is to release animals that will repopulate wild areas where Bd is present.

It has also been suggested that AMPs could be used as predictors of a species' susceptibility to Bd. The lack of anti-Bd AMPs was shown to correlate with declining populations in Bd afflicted Panamanian frog communities, suggesting that AMP profiles could be used as an indicator of susceptibility (Woodhams et al., 2006c). However, there are many susceptible species that have been found to produce AMPs in quantities sufficient enough to protect them from Bd infection (Parker et al., 2002, Rollins-Smith et al., 2002c, Woodhams et al., 2007a, Conlon et al., 2007a, Bosch and Rincón, 2008, Conlon et al., 2007b). These findings suggest that this defense alone is not capable of providing protection.

FUTURE RESEARCH:

The stronger correlation between chytridiomycosis resistance and anti-Bd skin microbes compared to AMPs provides evidence that bacteria may play a more important role in protecting frogs (Conlon et al., 2007b). It is even hypothesized that the reduction of AMP expression in some species is selected for to accommodate beneficial bacteria

(Conlon, 2011). It is also possible that the growth of skin microbes is regulated by AMPs (Küng et al., 2014) or that skin microbes enhance or mute the effects of AMPs; either way both defenses are likely engaged in a complex relationship. A combination of AMPs and a single bacterial metabolite (both from *R. muscosa* and both previously known to inhibit the growth of Bd individually) was more efficient at inhibiting the growth of Bd *in vitro* than either defense was alone (Myers et al., 2012). However, these laboratory assays do not represent the natural balance of AMPs and skin bacteria because only a bacterial metabolite was used.

Regardless, the relationship between AMPs and skin bacteria should not be overlooked and future research should focus on untangling this complex relationship. Rarely are the capabilities of both defenses analyzed within a particular frog species or between species that differ in susceptibility to chytridiomycosis. In one example where both were assayed, the resistant *R. sierrae* harbored more anti-Bd bacteria than the susceptible *R. muscosa,* but the abilities of their AMPs to inhibit the growth of Bd did not differ (Woodhams et al., 2007a). There are methods to independently remove each of these defenses from a frog, so future research should focus on manipulating each defense's presence while conducting susceptibility trials.

A large database consisting of the anti-Bd capabilities of susceptible and resistant species' AMPs and bacteria could support a meta-analysis that would help elucidate which defense is most important. This information would be even more useful if resolved to the population level, as different populations of the same species can vary in their compositions of AMPs and bacteria (Lam et al., 2010, Woodhams et al., 2010b, Holden et al., 2015).

When searching for mitigation strategies it is easy to focus only on the effect that the defense has on a pathogen and ignore the effect that a pathogen may have on a defense. Bd has been shown to impair the adaptive immune system in frogs (Fites et al., 2013b), and therefore could also impair their innate immune system. Recent evidence, from the laboratory and the field, suggests that Bd infection load can influence cutaneous bacteria compositions on *R. sierrae* by promoting the growth of certain bacterial taxa and inhibiting the growth of others (Jani and Briggs, 2014). Of the bacteria whose abundances were negatively correlated with Bd load, several belonged to genera that are known for their anti-Bd capabilities. However, a previous study examining the cutaneous bacteria of *R. sierrae* demonstrated that no strains belonging to these genera were capable of inhibiting Bd (Roth et al., 2013).

Still, this research provides evidence that at least some bacterial species belonging to these beneficial genera are sensitive to Bd, and that the impact of Bd infection on a frog's skin bacteria should not be overlooked. Similar laboratory and field studies should be conducted with other frog species whose compositions of anti-Bd and non-anti-Bd bacteria are known to determine how Bd infection load influences these compositions. Cutaneous bacteria can also be sampled and assayed against Bd before and after experimental infection of the host to determine if anti-Bd or non-anti-Bd bacteria change in response to infection. It would also be important to determine the mechanism that drives any observed changes in the bacterial community due to pathogen infection. These mechanisms could be direct (i.e. microbial composition) or indirect (i.e. a result of host behavior in response to infection). It is equally as important to examine the effects that Bd load can have on AMPs, although the natural brevity of

AMPs existence on the skin (approximately 2 hours [(Pask et al., 2012)]) may overshadow any observable effects.

CHAPTER 2

THE IDENTIFICATION OF ANTI-*BATRACHOCHYTRIUM DENDROBATIDIS* SKIN BACTERIA IN PERUVIAN MARSUPIAL FROGS

INTRODUCTION

The skin is arguably an amphibian's most important organ. This integument is responsible for most of the animal's respiration and osmoregulation. With form matching function, amphibian skin has a permeable design that facilitates diffusion and osmosis. Unfortunately this sensitive membrane also allows easy access for infectious pathogens so cutaneous defenses are especially important for amphibians. One particular pathogen, the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Longcore et al., 1999), invades amphibian skin with motile zoospores, which encyst and proliferate in keratinized dermal tissue (Berger et al., 2005a, Kilpatrick et al., 2010). Eventually, the functionality of the integument is interrupted with the onset of the disease chytridiomycosis (Berger et al., 2005b). This pathogen is of particular conservation concern because of its recent global spread and lethal effects (Stuart et al., 2004). Understanding the role of skin defenses in infection may lead to the development of novel mitigation strategies against Bd.

Amphibian skin is coated with a mucous consisting of a micro-environment, or mucosome, patrolled by components of both their adaptive and innate immune systems (Rollins-Smith et al., 2005). Protection is also provided by symbiotic bacteria (Harris et al., 2006). The bacteria that inhabit amphibian skin are likely established at a young age through environmental transmission from soil and water, although any sharing of refugia

is also likely to be an inter-individual source of inoculation, and for species with paternal care vertical transmission is probable (Walke et al. 2011, Fierer et al. 2012, Muletz et al. 2012). Amphibian skin often has a diverse microbial community (Jani and Briggs, 2014). Compositions are typically species-specific but can vary among populations within a species that is distributed over a wide geographic range (McKenzie et al., 2012, Lam et al., 2010). Some of these bacteria protect their hosts by secreting metabolites to destroy invading pathogens (Brucker et al., 2008) and may also outcompete pathogens for space on the skin (Belden and Harris, 2007).

Certain bacteria isolated from the skin of amphibians that are resistant to chytridiomycosis (henceforth, anti-Bd bacteria) have demonstrated the ability to inhibit the growth of Bd in *in vitro* co-culture assays (Flechas et al., 2012, Harris et al., 2006, Park et al., 2014). Metabolites isolated from cutaneous bacteria can also inhibit the growth of Bd alone (Loudon et al., 2014, Brucker et al., 2008), and it is suggested that zoospores actually avoid these metabolites through chemotaxis (Lam et al., 2011). Salamanders known to resist Bd infection experienced symptoms indicative of chytridiomycosis in experimental removals of anti-Bd bacteria (Becker and Harris, 2010). Additionally, the experimental addition of anti-Bd bacteria to susceptible amphibian species has ameliorated the effects of chytridiomycosis (Becker et al., 2009, Harris et al., 2009a, Harris et al., 2009b), suggesting that bioaugmentation therapy with probiotics may be a prophylactic strategy.

The inhabitants of an amphibian's skin appear to be highly regulated (Belden and Harris, 2007). A protocol to select anti-Bd bacteria for bioaugmentation suggests using bacteria already known to naturally inhabit the particular host species' skin (Bletz et al.,

2013), as not doing so has resulted in unsuccessful inoculation attempts (Becker et al., 2011) and could introduce bacteria that disrupt the natural bacterial communities. Therefore, the natural microbiome must be examined for any susceptible species intended to be treated by probiotics, requiring sampling across a diverse taxa. There is currently a database of bacterial strains known to inhibit Bd which includes their probable identities and their hosts (Woodhams et al., 2015). As the size of this database increases, the number of frog species it could help protect may also increase.

Although symbiotic bacteria are just one of many components of an amphibian's defensive mechanisms against pathogens, they are of particular importance for providing protection against Bd. Zoospores, the infectious life stage of Bd, must first penetrate an amphibian's skin to infect its host (Berger et al., 2005a), emphasizing the importance of the many innate defense mechanisms that patrol this integument. It is unclear if the adaptive immune system plays a vital role in protecting frogs from Bd (Berger et al., 1998a, Pessier et al., 1999, Berger et al., 2005b, Woodhams et al., 2007a). Bd may impair the development of adaptive cell-mediated defenses because experimental exposure of frogs to heat-killed Bd zoospores failed to induce a subsequent protective immune response (Stice and Briggs, 2010). The presence of lymphocytes does not increase in infected frogs (Woodhams et al., 2007a). It has even been demonstrated that Bd can impair lymphocytes via apoptosis in vitro (Fites et al., 2012) and may also impair a systemic immune response in vivo (Young et al., 2014). On the contrary, recent research does suggest that previously exposed frogs do acquire an immune response to Bd (McMahon et al., 2014), but only after several exposure and treatment regimens. Antimicrobial peptides (AMPs) have demonstrated the ability to

inhibit the growth of Bd *in vitro* (Conlon et al., 2013, Ramsey et al., 2010, Rollins-Smith et al., 2002c). However, even frog species susceptible to chytridiomycosis produce anti-Bd AMPs (Rollins-Smith et al., 2002c, Woodhams et al., 2007b, Bosch and Rincón, 2008, Parker et al., 2002, Conlon et al., 2007b),.

Amphibians are one of the most endangered vertebrate groups (Stuart et al., 2004), so developing mitigation strategies is critical. It is also unlikely that Bd will be eradicated from the environment, so mitigation strategies will rely on treatment or prophylactic methods directed at hosts. Although chytridiomycosis can currently be treated by the application of various anti-fungal drugs (Jones et al., 2012, Hadfield and Whitaker, 2005, Garner et al., 2009, Bowerman et al., 2010, Martel et al., 2011) or by heat therapy (Woodhams et al., 2003, Geiger et al., 2011, Chatfield and Richards-Zawacki, 2011), these treatments aren't always effective (Woodhams et al., 2012b) and would be difficult to apply *in situ*. Strategies that can protect vulnerable frogs may allow for populations to persist in their natural environment, and may improve understanding of how defenses like bacteria contribute to chytridiomycosis resistance and will guide future mitigation strategies.

We tested if cutaneous bacteria are important in protecting wild marsupial frogs in a community decimated by Bd in Andean Peru (Catenazzi et al., 2011). Female marsupial frogs possess a dorsal brood pouch where fertilized eggs are stored until hatching. Then, depending on the species, either tadpoles are deposited into water, or embryos complete their development inside the pouch and emerge as froglets (Weinland 1854(del Pino and Escobar, 1981). Recently, cutaneous bacteria were identified from Ecuadorian marsupial frogs that have tadpoles (Bresciano et al., 2015).

The species of focus in our study, *Gastrotheca nebulanastes* and *G. excubitor*, develop directly from egg to froglet (Duellman and Maness, 1980). Prevalence of Bd infection was similar in these species in 2008 and 2009 (Catenazzi et al., 2011).

A previous study exposed both *G. nebulanastes* and *G. excubitor* to high numbers of zoospores in Bd susceptibility trials. The results indicated that *G. nebulanastes* is susceptible to chytridiomycosis (16.6% survival) while *G. excubitor* is resistant (100% survival) (Catenazzi, Vredenburg et al. unpublished) (Figure 1). To test if cutaneous bacteria contributed to the observed difference in susceptibility, bacteria were collected from individuals of both species. Isolates of these bacteria were then tested for their abilities to inhibit the growth of Bd in co-culture assays. Growth inhibition was quantified by measuring the change in the density of Bd colonies. The identities of the isolates were also determined via BLAST searches of the sequences of their 16S ribosomal RNA genes, and bacterial compositions were compared between the two host species, as well as with other frog species (Woodhams et al., 2015, Bresciano et al., 2015).

METHODS

Study species and site

Frogs were collected along the Paucartambo-Shintuya road in the eastern slopes of the Cordillera de Paucartambo, Cusco, Peru from June to August of 2012. *Gastrotheca excubitor* were collected in high elevation grasslands above 3,000 m and *G. nebulanastes* were collected in the montane scrub forests between 2,000 and 3,000 m (Duellman et al., 2011). We captured juveniles and adults of both sexes. All frogs

were housed in individual plastic bags and taken to nearby Wayqecha Biological Station for bacterial and Bd sampling.

Bacterial Sampling

Samples of cutaneous bacteria were collected from 12 *G. excubitor* and 8 *G. nebulanastes*. Frogs were handled using sterile nitrile gloves and were rinsed twice with dechlorinated water to remove transient bacteria (Lauer et al. 2007). Frogs were then swabbed on their left, right, and ventral surfaces, hindlimbs, and interdigital membranes using a sterile cotton swab (Flechas et al. 2012). Each swab was streaked onto a Petri dish of nutritive agar and incubated at room temperature for up to two weeks. After this time Petri dishes were examined for bacterial growth and each bacterial morphotype (identified on the basis of color, form, elevation, and margin) was streaked onto a new plate with nutritive agar. This process was repeated until only similar morphotypes were consistently observed, at which time, a small portion of each isolate was collected onto a sterile cotton swab which was stored in a sterile vial. These were then transported to the laboratory for Bd growth inhibition assays, sequencing, and cryopreservation (nutritive broth with 30 % glycerol at – 80°C).

Batrachochytrium dendrobatidis growth inhibition assays

Each bacterial isolate was tested for its ability to inhibit the growth of Bd in a co-culture assay (Harris et al. 2006) at Universidad de los Andes in Bogotá, Columbia. We isolated the Bd strain used in these assays from the mouthparts of a *Hypsiboas gladiator*

(Anura, Hylidae) tadpole captured along the Paucartambo-Shintuya road in June of 2012. Bd was maintained in culture on TGh medium (10 g tryptone, 10 g agar, 4 g gelatin hydrolysate, 1 L distilled water) at 23˚C (Flechas et al. 2012). After maximum zoospore production was observed, Bd was harvested and combined in a sterile tube with 16 mL of sterile water. One mL of this zoospore rich solution was spread on to a Petri dish containing TGh medium and allowed to dry. An unknown isolate of bacteria from frog skin was streaked in a line across one side of the Petri dish, and as a negative control, *Escherichia coli* (strain DH5α), which does not inhibit the growth of Bd, was streaked across the other side of the Petri dish. Assays were set up in triplicate. Petri dishes were checked daily for contamination, and if a dish was contaminated it was removed and discarded. This was the reason for the removal of two replicates of one isolate and one replicate of another. Each Petri dish was grown at 23˚C for three days. On the third day each Petri dish was checked for Bd inhibition by the presence of a clear zone of inhibited Bd growth around the isolate.

Analysis of inhibition assays

The strength of a particular isolate's ability to inhibit Bd was determined by quantifying the distance from the isolate to where 50% Bd growth occurred. Thus, isolates with stronger inhibitory abilities depressed Bd growth over longer distances. For one replicate it was unclear where 50% growth occurred; the sigmoidal shape of the graphed points surpassed this value twice (Figure 3). This was likely due to sparse and uneven Bd growth on the Petri dish; this replicate was removed from analysis.

Bd growth was initially recorded by measuring the grey values of standardized photographs of the Petri dishes using ImageJ software (Abramoff et al., Flechas et al.,

2012). Grey values (hereafter referred to as “growth”) are measurements of the intensity of light in a pixel of a black and white image. Growth was averaged among pixel wide columns across the area between the query bacterium and negative control, thereby quantifying growth over minute distance increments from the query. Distances between the query and the negative control varied slightly between Petri dishes, so they were expressed as percentages of the total length (i.e. relative distance) to facilitate comparative analysis. Growth was also expressed as percentage of the maximum growth recorded to control for any variation between Petri dishes. For each replicate, values were plotted on a graph using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The distance at where 50% growth occurred was determined using the function of a line fit to the graphed values. We report average relative distance and standard error calculated across the three replicates of each isolate.

Identification of bacteria

Bacterial isolates were identified by sequencing the 16S ribosomal RNA (rRNA) gene. Swab samples of each isolate were stored frozen before processing. DNA was extracted from each swab using 80 μL Prepman Ultra Sample Preparation Reagent (Life Technologies Corporation, 2010). Tubes were then thoroughly vortexed, incubated at 100°C for 10 min., cooled for 2 min., and then centrifuged at 1300 rpm for 3 min. Afterwards, 40 μL of the supernatant was removed and placed into a new sterile tube. This DNA-rich liquid was diluted with sterile water at a ratio of 1:100. A PCR reaction was performed using the diluted DNA as a template and the universal 16S bacterial primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) (Lane, 1991). Each PCR reaction contained 1 μL of

each primer, 2 μL of the DNA template, 8.5 μL of sterile water, and 12.5 μL of GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA). Thermocycling conditions were as follows: initial denaturation at 95°C for 2 min., 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. Amplification was verified by electrophoresing 5 μL of the PCR sample on a 2% agarose gel. PCR products were cleaned using EXO-Sap It (Affymetrix, Inc, Santa Clara, CA, USA) and sent to MCLAB in San Francisco, California for sequencing.

Sequences were annotated and assembled using GENIOUS software (Biomatters Limited, Auckland, New Zealand). All sequences were first checked for quality and only those with forward and reverse sequences consisting of at least 60% high quality base pairs were utilized. Assembled sequences were referenced to known microbial DNA sequences using the BLAST search of the NCBI database within GENEIOUS using the default parameters. Sequences were also cross referenced with the Greengenes database (http://greengenes.lbl.gov) using the default parameters to verify identities. Identities of nearest relatives were approximated to either the genus or species level (depending on the results of the BLAST searches) based upon the identity grades of the BLAST results.

Sampling for Bd

All individuals collected during the field sampling were swabbed for the presence of Bd. Sterile cotton swabs were streaked five times on each side of the animal, between its front and rear limbs, five times on the ventral “drink patch,” five times on the upper portion of each of the legs, and once on each interdigital membrane of the hind

feet (Hyatt et al., 2007). The swabs were immediately placed into individual sterilized vials and stored for processing.

Swab samples were analyzed using real-time quantitative PCR (qPCR). DNA was extracted from swabs using PrepMan Ultra following the protocol outlined in Boyle et al. (2004) and Hyatt et al. (2007), except extracts were analyzed once instead of three times (Vredenburg et al., 2010). An Applied Biosystems Instrument 7300 PCR machine was used to quantify the amount of DNA in each sample. We calculated ZE, the genomic equivalent for Bd zoospores (henceforth "zoospores") by comparing the qPCR results to a set of standards from live cultures.

RESULTS

Identification of bacteria via morphology and RNA sequencing

A total of 24 bacterial morphotypes were isolated form the study species, 14 from *G. excubitor* and 10 from *G. nebulanastes* (Table 1). The nearest relatives for three of the anti-Bd strains (see below) isolated from *G. excubitor* were: *Sphingobacterium faecium* (99.7% identity grade)*, Janthinobacterium lividum* (99.9% identity grade), and *Rahnella aquatilis* (99% identity grade) and were labeled as strains of these species*.* The other two isolates from *G. excubitor* were identified as members of the genus *Pseudomonas,* but because they lacked a clear nearest relative on the species level were designated with the labels *Pseudomonas* sp. 1 and *Pseudomonas* sp. 2. The nearest relatives for *Pseudomonas* sp. 1 are *P. mandelii, P. poae,* and *P. trivialis* (all 99.4% identity grades). The nearest relatives for *Pseudomonas* sp. 2 are *P. fluorescens,*

P. poae, and *P. trivialis* (all 100% identity grades). These similar BLAST results indicate that these two isolates are probably closely related. However, they are morphologically distinct in culture.

The single anti-Bd bacterium isolated from *G. nebulanastes* also belongs to the genus *Pseudomonas*. Due to ambiguity with identifying this isolate's nearest relative on the species level, it was labeled *Pseudomonas* sp. 3. The two nearest relatives of *Pseudomonas* sp. 3 (100% identity grade) are also currently unidentified to the species level, but they are closely related to *P. baetica* and *P. koreensis* (99.9% identity grade).

Among non-anti-Bd isolates (see below), one isolated from *G. excubitor*, had the same sequences of *Pseudomonas* sp. 2, suggesting variability in this isolate's Bd inhibitory abilities. Six others from *G. nebulanastes* all had many nearest relatives within the genus *Psuedomonas*, including some that were shared with the *Pseudomonas* anti-Bd isolates such as *P. poae* (99.9% identity grade), *P. fluorescens* (99.9% identity grade), and *P. baetica* (100%). The nearest relatives for several non-anti-Bd isolates were identified to the species level, including *Microbacterium oxydans* (99.9 %) and *Bacillus simplex* (100% identity grade) from *G. nebulanastes*, and *Aeromonas hydrophila* (100%) and *Acinetobacter radioresistens* (99.9% identity grade) from *G. excubitor*.

Batrachochytrium dendrobatidis growth inhibition assays

From the inhibition assays, six out of the total 24 bacterial isolates tested produced zones of Bd inhibition. Of these, five came from *G. excubitor* and one from *G. nebulanastes*, so about a third of *G. excubitor* (five out of 14) isolates but a tenth of *G. nebulanastes* isolates (one out of 10) were labeled as anti-Bd (figure).

Analysis of the inhibition assays indicated that the bacterium labeled as *Pseudomonas* sp. 2, isolated from *G. excubitor*, had the strongest ability to inhibit the growth of Bd, with 50% Bd growth measured occurring at 66% of the total distance (Figure 4, Table 1). The *J. lividum* strain had the weakest inhibitory ability of all of the bacteria isolated from *G. excubitor*. For this isolate, 50% of Bd growth occurred at 40% of the total distance (Figure 4). The one isolate from *G. nebulanastes* that was inhibitory against Bd, *Pseudomonas* sp. 3, was the weakest of all the isolates from both species. For this isolate, 50% of Bd growth occurred at 38% of the total distance (Figure 4).

Prevalence and infection load for Bd

Only four *G. nebulanastes* (prevalence= 25%, CI=7.6%-46.8%) but 13 *G. excubitor* (prevalence= 46.4%, CI=28.9%-64.3%) tested positive for Bd. All Bd positive frogs had low infection loads (< 350 zoospore), except for one *G. excubitor*, with 26,208.8 zoospore.

DISCUSSION

The frog species resistant to chytridiomycosis, *G. excubitor*, harbors more cultivable anti-Bd skin bacteria than a susceptible congeneric, *G. nebulanastes*. *Gastrotheca excubitor* also has proportionally more cultivable bacteria that inhibit the growth of Bd than *G. nebulanastes* (36% versus 10%, respectively). These results suggest that chytridiomycosis resistance may be correlated with the number and proportion of a frog species' anti-Bd skin bacteria. This relationship has also been

suggested by other studies that found similar results (Park et al., 2014, Woodhams et al., 2007b, Lam et al., 2010, Lam et al., 2008, Flechas et al., 2012).

All of the six anti-Bd bacterial morphotypes had relatively similar strengths of inhibition with distances to 50% Bd growth ranging from 38%-66% of the total distance (Figure 4). The isolate with the strongest ability to inhibit Bd belongs to the genus *Pseudomonas* (Figure 4). Two other isolates were identified as members of this genus, the fourth strongest and the weakest (Figure 4, Table 1). *Pseudomonas* bacteria are common inhabitants of frog skin and are frequently characterized by having strong anti-Bd properties (Flechas et al., 2012, Walke et al., 2011, Brucker et al., 2008, Lam et al., 2010). The nearest relatives of two isolates, *S. faecium* and *J. lividum*, are known for their anti-Bd abilities; for example *S. faecium* has been isolated from *Alytes obstetricans* in Switzerland, a toad species whose susceptibly to Bd varies by population (Tobler and Schmidt, 2010).

The presence of *J. lividum* on the skin of *G. excubitor* is of particular interest. Anti-Bd strains of this bacterium have been isolated from multiple species of amphibians from Europe and the United States (Woodhams et al., 2015). This bacterium has also been applied successfully as a probiotic in laboratory experiments (Harris et al., 2009b, Harris et al., 2009a) and in one emergency application to a declining population of *Rana muscosa* in the wild (Vredenburg et al., 2011), although the latter experiment did not have a control group. Despite *J. lividum* being well-known for the strong anti-Bd properties of its metabolites violacein and indole-3-carboxaldehyde (Brucker et al., 2008), the closely related strain that we isolated was ranked as the second weakest out of six isolates (Figure 4). Our strain also lacks the distinctive purple coloration

characteristic of *J. lividum* in culture, attributed to the production of violacein. It is possible that our strain does not produce violacein but still produces indole-3-carboxaldehyde, making it less able to inhibit Bd than other *J. lividum* strains.

Although not previously known for its ability to inhibit the growth of Bd, we found that *Rahnella aquatilis,* isolated from *G. excubitor*, was a relatively strong inhibitor of Bd (Figure 4). This bacterium has been documented as an antifungal symbiont of the Chinese beetle *Dendroroctonus valens* (Winder et al., 2010). The two isolates from *G. excubitor* in the genus *Pseudomonas* were both closely related to *Pseudomonas poae* (99.4% identity grade for *Pseudomonas* sp. 1 and 100% identity grade *Pseudomonas* sp. 2), documented as an antifungal symbiont of the sugar beet (Müller et al., 2013). Although both the isolates *Pseudomonas* sp. 1 and *Pseudomonas* sp. 2 had similar 16S rRNA sequences, their morphologies were distinct and they differed in their abilities to inhibit Bd (with 50% Bd growth occurring at 43.33% and 65.94% of the total distances, respectively). We found that a non-anti-Bd isolate was also identified as *Pseudomonas* sp. 2 because of their identical 16S rRNA sequences. Additionally, several non-anti-Bd *Pseudomonas* isolates shared similar nearest relative BLAST results to these anti-Bd *Pseudomonas* strains. These findings suggest that functional variance can occur between closely related, or even same-species, strains.

The one anti-Bd strain from *G. nebulanastes*, labeled as *Pseudomonas* sp. 3, shares a 100% identity grade with a bacterium isolated from peat moss in the United Kingdom (Hill et al., 2014) and another, capable of producing aromatic compounds, isolated from soil underneath logs in the Netherlands (Bandounas et al., 2011). The latter is closely related to another bacterium with documented antifungal properties; it is

pathogenic to the mushroom *Agaricus bisporus* (Godfrey et al., 2001). Although labeled as anti-Bd on the basis of its inhibition zone in one replicate, the removal of the second replicate (see methods) brings into question the integrity of its inhibitory abilities. Therefore, we label *Pseudomonas* sp. 3 as potentially anti-Bd and suggest further testing to verify its anti-Bd ability.

None of the bacteria identified were shared between the two *Gastrotheca* species. These frogs differ in the niches that they inhabit, with *G. excubitor* dwelling under rocks and among bunchgrasses on the ground of grasslands and *G. nebulanastes* in the bushes and trees of the scrub forest. Our findings suggests that the environment might be an important source of inoculation for skin bacteria. We also found that only *J. lividum* and *Aeromonas hydrophila*, isolated from *G. excubitor*, were shared with the Ecuadorian congeneric *G. riobambae* sampled by Bresciano et al. (2015). Interestingly, this study considered *A. hydrophila* as anti-Bd, and we did not. However, unlike our study, Bresciano et al. (2015) did not conduct growth inhibition assays to validate the classification of their bacteria. This difference, along with our varying results for *Pseudomonas* sp. 2, exemplifies the importance of considering the variance in the antimicrobial abilities of closely related strains.

The results of our study support the notion that cutaneous bacteria may be important in providing frogs with protection against Bd (Conlon et al., 2007b). Previously, it had been suggested that frogs fail to develop an adaptive immune response (Stice and Briggs, 2010) and that proliferation of lymphocytic cells is hindered by Bd (Fites et al., 2012). In addition, the expression of AMPs does not consistently correlate with resistance to Bd (Rollins-Smith et al., 2002c, Woodhams et al., 2007b, Bosch and

Rincón, 2008, Parker et al., 2002, Conlon et al., 2007b), and the reduction of these defenses has not been demonstrated to increase susceptibility (Woodhams et al., 2012a) as does the reduction of anti-Bd skin bacteria (Becker and Harris, 2010). A concurrent study conducted by our group found that natural mixtures of AMPs collected from *G. excubitor* and *G. nebulanastes* did not differ in their abilities to inhibit the growth of Bd *in vitro*, emphasizing the role of the anti-Bd bacteria found here in protecting *G. excubitor*.

The use of anti-Bd bacteria in bioaugmentation therapy is promising, but the strict requirements of potential probiotics (Bletz et al., 2013) will require wide scale sampling of cutaneous microbes from many frog species. Here we have discovered at least four novel anti-Bd strains that will contribute to the library of these potential candidates. We did not, however, find any anti-Bd isolates shared between the *Gastrotheca* species, which suggests that similarities in cutaneous bacterial compositions cannot be assumed between congeneric species. Bletz et al. (2013) recommends as probiotic a bacterium that naturally occurs on the susceptible frog. In our study, the only candidate for bioaugmentation therapy for *G. nebulanastes* is *Pseudomonas* sp. 3, whose anti-Bd capabilities are questionable. If its inhibitory abilities are eventually verified, however, this bacterium should be considered as a candidate for bioaugmentation therapy with *G. nebulanastes.* Since the females of this species keep eggs in their dorsal brood pouch until the hatching of froglets (Duellman et al., 2011), vertical transmission of cutaneous bacteria between a mother and offspring is likely. Therefore, a probiotic applied to a portion of wild females of this species may proliferate throughout generations.

CONCLUSIONS:

The process towards developing an effective mitigation strategy for chytridiomycosis is an iterative approach that continues to unveil the complexity of the host-pathogen system. It is likely that bacteria may provide an answer and the results of our research are encouraging. The intricate ecology of the frog mucosome must first be untangled to know for certain. Outside the scope of the frog's immune system, inter- and intraspecific interactions, environmental factors, and anthropogenic forces likely play major roles in the patterns of either post-Bd population declines or persistence. Despite this daunting complexity, however, each discovery is a step forward towards understanding the host-pathogen relationship and brings hope for saving an imperiled animal group.

Table 1: Species of cultured bacteria isolated from *Gastrotheca excubitor* and *G. nebulanastes*

Bacterial Species	***G. exc.***	***G. neb.***	**Anti-Bd**	**Rank of anti-Bd**
Actinobacteria				
Actinomycetales: Microbacteriaceae				
Microbacterium oxydans	-	+	No	
Microbacterium sp.	+	-	No	
Bacteroidetes				
Flavobacteriales: Flavobacteriaceae				
Chryseobacterium sp.	+	-	No	
Sphingobacteriales: Sphingobacteriaceae				
Sphingobacterium faecium	+	-	Yes	3
Firmicutes				
Bacillales: Bacillaceae				
Bacillus simplex	-	+	No	
Bacillus sp.	-	+	No	
Bacillales: Paenibacillaceae				
Paenibacillus sp.	-	+	No	
Proteobacteria				
Aeromonadales: Aeromonadaceae				
Aeromonas hydrohphila	+	-	No	

Table [1]: Continued

Bacterial Species	***G. exc.***	***G. neb.***	**Anti-Bd**	**Rank of anti-Bd**
Burkholderiales: Oxalobacteraceae				
Janthinobacterium lividum	+	-	Yes	5
Enterobacteriales: Enterobacteriaceae				
Rahnella aquatilis	+	-	Yes	2
Serratia sp.	+	-	No	
Pseuodomonadales: Moraxellaceae				
Acinetobacter radioresistens	+	-	No	
Pseudomonadales: Pseudomonadaceae				
Pseudomonas sp. 1	+	-	Yes	4
Pseudomonas sp. 2	+	-	Yes	1
Pseudomonas sp. 3	-	+	Yes	6
Pseudomonas sp. 4	-	+	No	
Pseudomonas sp. 5	-	+	No	
Pseudomonas sp. 6	-	+	No	
Pseudomonas sp. 7	-	+	No	
Pseudomonas sp. 8	-	+	No	
Sphingomonadales: Sphingomonadaceae				
Sphingomonas sp.	+	-	No	

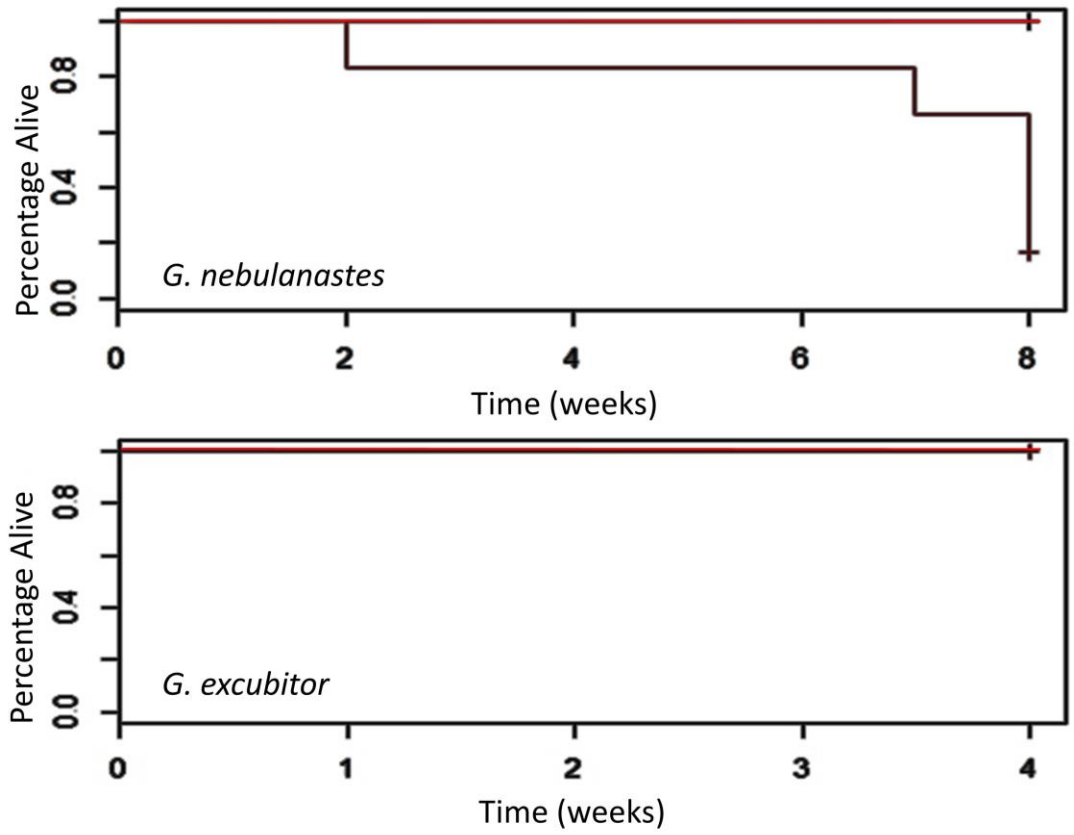

Figure 1: Kaplan-Meier Survival Curves for *G. nebulanastes* (top) and *G. excubitor* (bottom). The infected group is represented in black and the control group in red. Infected and control *G. excubitor* did not differ in survivorship.

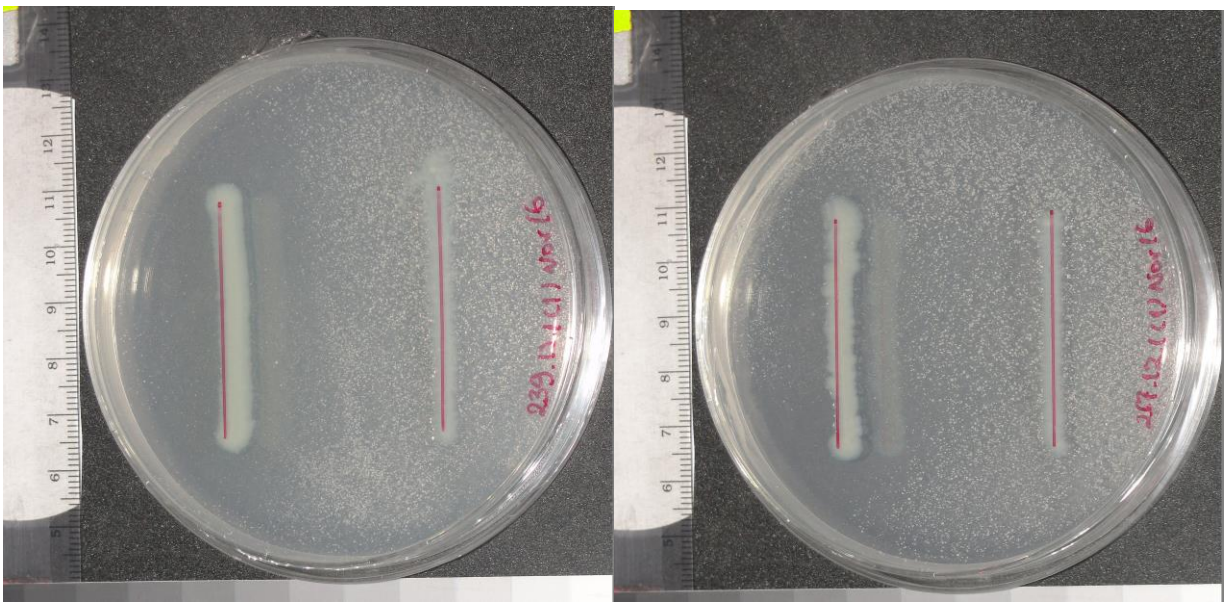

Figure 2: Examples of bacterial isolates exhibiting clear zones of Bd inhibition and therefore designated as anti-Bd.

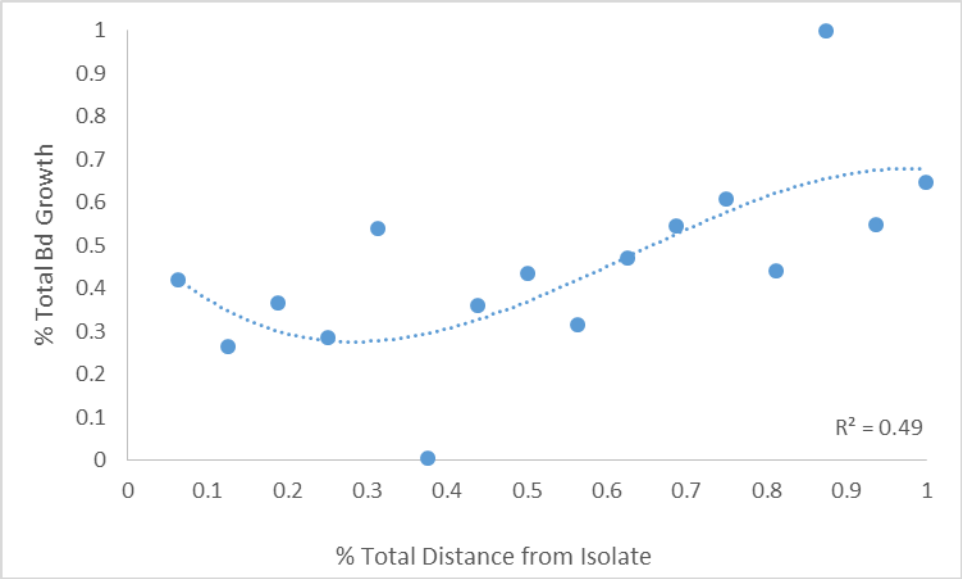

Figure 3: The growth inhibition curve for a replicate of the *Pseudomonas* sp 3 isolate. Because the line of this graph passes through 50% growth (0.32% total Bd growth based on the equation of the line) twice, it was excluded from further analysis.

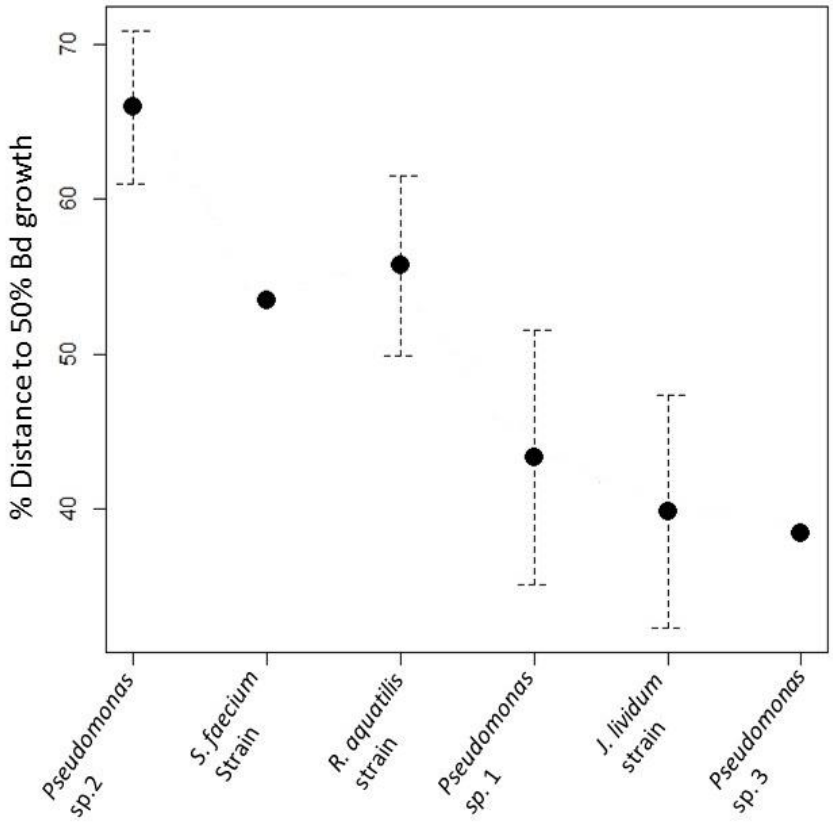

Figure 4: Mean distances (with standard errors) to 50% growth for each isolate. *Sphingobacterium faecium* and *Pseudomonas* sp. 3 do not have standard error bars because they each had only 1 replicate.

CHAPTER 3

SUSCEPTIBILITY TO CHYTRIDIOMYCOSIS DOES NOT CORRELATE WITH ANTIMICROBIAL ABILITIES OF CUTANEOUS PEPTIDES IN TWO SPECIES OF MARSUPIAL FROGS

INTRODUCTION

Antimicrobial peptides (AMPs) are an important component of the innate immune system for many organisms including both vertebrates and invertebrates. AMPs are functionally diverse but all share the ability to form an amphipathic shape characterized by distinct hydrophobic and cationic amino acid groups. This structure allows AMPs to efficiently target cellular membranes and induce lysis (Amiche et al., 1999, Conlon et al., 2004b, Pasupuleti et al., 2012). An AMP's positive charge facilitates binding to bacterial or fungal outer membranes which are composed of many negatively charged phospholipids (Nicolas and Mor, 1995, Zasloff, 2002). Because of its hydrophobicity, an AMP can also penetrate its target's cellular membrane, creating fatal pores (Yang et al., 2000). It is suggested that cellular death is induced by depolarization of the membrane (Westerhoff et al., 1989), reorganization of the lipid bilayer (Matsuzaki, 1999), or by the peptide entering the cell and disrupting enzymatic activity (Kragol et al., 2001).

Evolutionarily, AMPs are an ancient defense mechanism and are therefore probably important for basal vertebrates like amphibians. Invasion of pathogens through porous skin is problematic for these animals, emphasizing the efficiency of cutaneous defenses like AMPs. It is suggested that AMPs may even assist with the delivery of neuroactive peptides in toxic amphibians by disrupting the cellular membranes of their

predators (König et al., 2015, Conlon, 2011). Because they can be proficient killers, especially in anurans, amphibian AMPs have been tested for biomedical applications, demonstrating activity against *Chlamydia trachomatis* (Bergaoui et al., 2013), human immunodeficiency virus (VanCompernolle et al., 2005), and tumor cells (Mechkarska et al., 2014).

In frogs, activation of the sympathetic nervous system stimulates cutaneous granular glands to release AMPs which are combined with a mucous to coat the skin (Bovbjerg, 1963, Mills and Prum, 1984, Rollins-Smith et al., 2005). Since they are present on the skin, AMPs could play a role in protecting frogs from cutaneous pathogens like the fungus *Batrachochytrium dendrobatidis* (Bd) (Longcore et al., 1999). This pathogen has caused numerous frog population collapses in many parts of the world (Berger et al., 1998a, Daszak and Berger, 1999, Skerratt et al., 2007, Fisher et al., 2009). Bd infects frogs via motile zoospores that encyst on their skin (Berger et al., 2005a, Kilpatrick et al., 2010) and can cause the fatal disease chytridiomycosis (Berger et al., 2005b).

Natural peptide mixtures collected from frogs have successfully demonstrated the ability to inhibit the growth of Bd in laboratory assays (Rollins-Smith et al., 2002c, Conlon et al., 2013, Woodhams et al., 2010a, Ramsey et al., 2010). The collection of peptides is typically induced by injecting the frog with norepinephrine to stimulate its sympathetic nervous system (Rollins-Smith et al., 2002c, Nutkins and Williams, 1989). Bd growth inhibition is then assayed using different dilutions of natural peptide mixtures to establish a minimum inhibitory concentration (MIC) where growth of a known number of zoospores is completely inhibited (Woodhams et al., 2006b, Rollins-Smith et al.,

2006). MIC values can be used to compare the relative effectiveness of AMPs among individuals or species.

Here, we analyze peptide samples from two marsupial frogs, *Gastrotheca excubitor* and *G. nebulanastes*. Whole peptide mixtures collected from these frogs were tested for their abilities to inhibit the growth of Bd *in vitro* and the peptide profile of each sample was generated by mass spectrometry. The results contribute to understanding the role that AMPs play in protecting frogs from chytridiomycosis because *G. excubitor* is resistant and *G. nebulanastes* is susceptible (Catenazzi, Vredenburg et al. unpublished). These species live in the Andes Mountains of south eastern Peru, where surveys indicate that anuran species diversity was reduced by approximately 36% following the arrival of Bd at the turn of the millennium (Catenazzi et al., 2011).

METHODS

Study species

Marsupial frogs (family Hemiphractidae) occur from Central America to southern South America. These frogs are unique in that the females possess a dorsal brood pouch where eggs develop until hatching as either a tadpole or a froglet (direct-developing) depending on the species (Weinland 1854, del Pino and Escobar, 1981). Both *G. nebulanastes* and *G. excubitor* are medium-sized, direct-developing frogs endemic to the Andean region of southeastern Peru. These two species are morphologically similar and were thought to be the same species until 2011 (Duellman et al., 2011). The susceptible *G. nebulanastes* inhabits montane scrub and cloud forests

from 2,000-3,300 m. They are primarily found on shrubs and the rocks of cliffs caused by landslides or along roads (Duellman et al., 2011). The resistant *G. excubitor* is marginally sympatric to *G. nebulanastes* but lives above 3,100 m on vegetation in high-Andean grasslands.

Study site

Field work was conducted in the Kosñipata Valley of southeastern Peru. Surveys indicated that the number of frog species present in the montane area (1200 – 3800 m) has declined by approximately 36% from 1999 to 2008 and 2009, and is significantly correlated with prevalence of Bd along the elevational gradient (Catenazzi et al., 2011). Both *G. nebulanastes* and *G. excubitor* can be found near the Wayqecha Biological Station (WBS) located at 2900 m on the mountainside of the valley. This research station is just outside of Manu National Park in the region of Cusco. Its property encompasses cloud forests, montane scrub forests, and grasslands from 2200 m to 3700 m. The Paucartambo-Shintuya Road runs through these ecosystems and provides easy access.

Collection of frogs

Visual encounter surveys performed from May to July of 2014 recovered 36 *G. nebulanastes* and 47 *G. excubitor* in the area surrounding WBS. *Gastrotheca nebulanastes* were collected by walking along the Paucartambo-Shintuya Road at night, where males were found calling from rocks and vegetation and juveniles were foraging. No females were encountered during the sampling period. Previously recorded calls were played to maximize the chance of locating calling males. *Gastrotheca excubitor*

were collected during the day by searching under rocks, in bunchgrasses, and in moss in the high-elevation grasslands they inhabit. Males, females and juveniles of this species were all commonly encountered and therefore all were included in the sampling.

Frogs were collected by inverting a plastic bag over the collector's hand, reducing any risk of contamination from the collector's skin (Robertson et al., 2013, Woodhams et al., 2006a). A small piece of nearby vegetation, collected in the same manner, was placed in each bag to provide moisture for the frog. Following a survey, all collected frogs were brought back to WBS, stored in their individual bags in a cool location away from direct sunlight, and sampled for peptides within 24 hours. Afterwards they were released in the area at which they were found.

Sampling for Antimicrobial Peptides

Peptide secretion was stimulated by the injection of norepinephrine into each frog's dorsal lymph sack (Rollins-Smith et al., 2002c, Nutkins and Williams, 1989). Norepinephrine injection provides a more standardized peptide collection technique alternative to transdermal electrical stimulation, which requires calibration between species and is thought to be more harmful (Tyler et al., 1992, Woodhams et al., 2006a). Specifically, 0.1 ml of injection solution prepared as described below (Holden et al., 2015) was administered per gram of frog body weight (Table 2). To obtain this concentration in the field, pre-weighed quantities (0.675 mg) of norepinephrine were combined with 5 ml of amphibian phosphate buffered saline (APBS, Table 2) and mixed by vortex to produce a 40 nmol solution. This solution was then passed through a 0.22 μm filter to sterilize it before use. A fresh batch of injection solution was made before each AMP sampling.

Prior to injection, the frog's entire body was first rinsed for five seconds with deionized water to remove any transient contaminants and dirt from its skin. The frog was then placed into a sterile Whirl- Pak® bag (Nasco, Fort Atkinson, WI, USA) and weighed to determine the volume of injection solution to administer. The frog was manipulated to the top of the bag (without making contact with it) and the norepinephrine solution was injected through the bag and into the frog's dorsal lymph sack using a 1 mL syringe with a new, sterile 5/8 inch 25 gauge needle for each frog. Immediately after administering the injection solution, 25 ml of HPLC grade water was added to the bag as a medium for peptide collection. The frog remained in the bag for 15 min, enough time to allow the maximum amount of skin secretions to collect in the water before the peptides may begin to degrade (Rollins-Smith et al., 2002c).

After 15 min, the frog was removed from the bag and 1 ml of 50% HCl (Table 2) was added to the sample (Woodhams et al., 2006b) to inactivate any endogenous peptidases that may be present (Resnick et al., 1991, Steinborner et al., 1997). The samples were partially purified by passing them through Sep Pak cartridges (Waters Corporation, Milford, MA, USA) (Goraya et al., 1998) that had previously been activated by passing 10 mL of ethanol and then 10 mL of Buffer A (Table 2). Using a 60 mL syringe, the entire volume of the collection water and skin secretions was collected from the bag, pushed through a single Sep Pak cartridge at a rate of two drops per second and collected in a waste container (Louise Rollins-Smith, pers. comm.). The cartridges were then placed back in the Whirl-Pak® storage bags and kept with 2 mL of Buffer A to stay moist until processing. Meanwhile, the frog was swabbed for Bd (see below), measured for snout to vent length, and sexed.

Elution of peptides from skin secretion samples

After collection, the skin secretion samples were transported to Vanderbilt University and peptides were eluted from the Sep Pak cartridges by Laura Reinert in the Rollins-Smith lab. Elution was achieved by passing the samples through a vacuum pump set to 3 rpms. First, 10 mL of Buffer A were passed through the cartridges and into a waste container to remove any unwanted material that may have been lodged in the filter. Then, each cartridge was flushed with 11 mL of Buffer B, containing acetonitrile which liberated the bound peptides. The resulting solution was collected in sterile, individual 50 mL conical tubes. One mL from each sample was removed and placed into a mini Eppendorf tube and stored at -80˚ C for peptide quantification (see below). The remaining 10 mL of each sample was vacuum-centrifuged to dryness. The lyophilized peptides were then re-suspended in HPLC water, each at a concentration of 1 mg/mL (based on the quantity recovered per sample as determined by microBCA protein assays, see description below) for composition analysis (Louise Rollins-Smith, pers. comm.).

Quantifying peptides in skin secretion samples

Peptide concentrations per each sample were quantified using a Pierce microBCA protein assay kit (Thermo Scientific, Rockford, Illinois). The assay tests 1:10 dilutions and non-diluted skin secretion samples against standards (200, 40, 20, 10, 5, 2.5, 1, and 0.5 µg/mL). The manufacturer's protocol was followed, except the peptide bradykinin (RPPGFSPFR) was used to determine the standards (Rollins-Smith et al., 2002c, Smith et al., 1985). Solutions were plated on a 96-well plate with the standards

and blanks (Buffer B only) as negative controls, all in triplicate to increase the accuracy of comparisons (Louise Rollins-Smith, pers. comm.). Next, the plate was incubated at 37˚C for two hours then cooled to room temperature for 10 minutes. The absorbance of each well was analyzed using an absorbance microplate reader at 570 nm. The results for each sample were compared with the standards to determine a peptide quantity per sample. One juvenile *G. excubitor* sample was removed from analysis because it contained no peptides.

Bd growth inhibition assays

Whole peptide mixtures were tested *in vitro* by dilution assays for their ability to inhibit the growth of Bd zoospores (JEL 197, J. E. Longcore, University of Maine). Only individual samples with enough peptide mixtures after resuspension (at least 600 μL at 1 mg/mL) to complete an assay were tested for growth inhibition (n= 9 for *G. excubitor* and 8 for *G. nebulanastes*). Bd was maintained at 19˚C in flasks with 1% tryptone broth containing penicillin and streptomycin to inhibit bacterial growth. Only zoospores were used because they are the infectious propagules of Bd (Rollins-Smith et al., 2002a). Zoospore harvesting was initiated by first scraping the sides of flasks (containing at least one week of growth, indicated by the presence of flakes of mature thalli) with a sterile inoculation loop to remove any zoospores that were against the sides. Two mL of solution was then removed from these flasks, flooded onto agar plates (tryptone, agar, glucose), and dried under a laminar flow hood. These plates were then incubated at 19-20˚C until swarms of active zoospores were visible, typically three to seven days. Zoospores were harvested by flushing plates with tryptone broth and then passing the liquid through a 20 μm filter in a Buckner Funnel attached to a vacuum flask. This filter

size traps the mature thalli but allow zoospores to pass. The resulting zoospore-rich broth was then transferred from the vacuum flask to a sterile 50-ml conical tube. A sample of this solution, diluted in broth (1:10), was removed and placed onto a hemocytometer to count zoospores. This value was used to dilute the original zoospore rich solution to a concentration of $1x10^6$ zoospores/ml.

The peptide-Bd inhibition assays were conducted on 96-well microtiter flat-bottom plates, one plate per an individual frog's peptide sample. Each well contained a total of 100 µL; the assay consisted of five replicates each of: positive controls for Bd growth (50 µL zoospore solution and 50 µL HPLC water), negative controls for Bd growth (50 µL zoospore solution, placed in a 60 ˚C water bath for 10 min to heat kill zoospores, and 50 µL HPLC water), and peptide dilutions at 500, 250, 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL of whole peptide mixtures (50 µL peptide solution and 50 µL zoospore solution). Because plating of the assay resulted in diluting the zoospore solution 1:2, the final concentration tested for growth inhibition was $5x10^5$ zoospores/ml. The absorbance (A) at 490 nm (A_{490}) (Rollins-Smith et al., 2002c) was immediately recorded of each well using a Fisher Scientific accuSkan GO UV/VIS microplate spectrophotometer (Fisher Scientific, Hampton, NH, USA). Plates were then covered by a lid, sealed with Parafilm (Bemis NA, Neenah, WA, USA), and incubated at 19-20˚C for seven days. On the seventh day, the A_{490} was recorded again. Plates were regularly checked for contamination by visual inspection, and any plates or wells that were contaminated were excluded from analysis. Any assays with more than three excluded wells within a treatment were removed from analysis.

Peptide Composition analysis via MALDI TOF mass spectrometry

Peptide composition profiles were obtained for each individual frog sample (n= 36 for *G. nebulanastes* and 47 for *G. excubitor*) via matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (Woodhams et al., 2006b, Rollins-Smith et al., 2006). Mass spectrometry was performed at SIUC on a Thermo Scientific TraceGC PolarisQ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in linear mode. The instrument was calibrated using the following standards (Sigma Aldrich, St. Louis, Missouri USA): bradykinin (m/z 904.468), angiotensin I (m/z 1296.685), Glu-Fibrinopeptide B (m/z 1570.677), ACTH 1-17 (m/z 2093.085), ACTH 16-39 (m/z 2465.199), and insulin oxidized B chain (m/z 3494.6513). Combined standards were mixed with matrix (10mg/mL α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 49.9% MilliQ water, 0.1% trifluoroacetic acid) at a ratio of 1:25 and peptide samples were mixed with matrix at a ratio of 1:1. Both were spotted onto a MALDI plate, alternating rows between samples and standards so that every sample spot was adjacent to a standard spot.

Sampling for Bd

All individuals collected during the cutaneous peptide sampling were sampled for the presence and quantification of Bd infection. For each frog, a sterile cotton swab was streaked five times each on the side, between the front and rear limbs, the ventral pelvic patch, the upper portion of each of the legs, and once on each interdigital membrane of the hind feet (Hyatt et al., 2007). The swabs were immediately placed into individual sterilized vials until processing by real time PCR. PrepMan Ultra was used to extract

DNA from the swabs using the protocol outlined in Boyle et al. (2004) and Hyatt et al. (2007), except extracts were analyzed once (Vredenburg et al., 2010). Real time PCR was carried out on an Applied Biosystems StepOnePlus PCR machine to calculate the genomic equivalent, ZE for Bd zoospores (henceforth "zoospores"). This method quantifies these genomic equivalents by comparing each sample to a set of diluted standards from 100 to 0.1 genomic equivalents of zoospores.

Statistical Analysis

All statistical analyses were completed using R version 3.0.3 (R Foundation). Peptide amounts recovered were log transformed to meet the requirements of normality. These values were then compared between species and sex and life stage (includes males, females, and juveniles) using a two-way analysis of variance (ANOVA) with Tukey's post hoc test (n=3 for *G. nebulanastes* juveniles, 33 for *G. nebulanastes* males, 22 for *G. excubitor* juveniles, 12 for *G. excubitor* males, and 12 for *G. excubitor* females). Additionally, the amounts of peptides were compared between sex and life stage within *G. excubitor* (to look for differences among groups within a single species) using a one way ANOVA with Tukey's post hoc tests. This analysis was not done for *G. nebulanastes* because, besides three juveniles, only males were sampled.

Data from inhibition assays were normally distributed (indicated by plotting quantiles), thus outliers were removed from analysis after using Dixon's Q test (Holden et al., 2015). Positive control wells with no Bd growth and negative control wells with Bd growth were also excluded from analysis. For each well, the A_{490} from the first day was subtracted from that of the seventh day to determine ΔA_{490}. Mean ΔA_{490} for the five negative control wells was then subtracted from every well to account for any change in

A_{490} not attributed to Bd growth. These resulting values were then taken as a percentage of mean ΔA_{490} for the positive control wells to standardize measurements across assays for statistical comparisons. The resulting percentages ($p\Delta A_{490}$) represented Bd growth in each experimental well after the seven day period. $p\Delta A_{490} < 1$ represented Bd growth inhibition, while $p\Delta A_{490} > 1$ represented enhanced Bd growth. The abilities for individual peptide mixtures to inhibit the growth of Bd were visualized graphically by plotting the average percent of Bd growth for the different peptide concentrations and the controls (Figures 6-7). For a peptide mixture to be considered inhibitory it had to both significantly inhibit Bd growth at high concentrations and also exhibit an overall negative correlation with Bd growth as concentration decreased.

Due to the design of the experiment, the amount of Bd growth among peptide dilutions and between species was compared by a split plot ANOVA design (Figure 10). The design treated individual frogs as a random effect nested within species and used the average amount of growth (the average of the five replicates) for each concentration within an individual. This design facilitated the statistical comparison of both frog species and dilution as factors in a single analysis. The model was adjusted with a Kenward-Roger Degrees of Freedom Approximation and paired with a Tukey's post hoc test.

Peptide profiles generated by MALDI TOF mass spectrometry were analyzed using the software Flex Analysis (Bruker, Billerica, MA, USA) to mark the masses in Daltons (m/z) and intensities (counts per second) of peptide signals. The occurrence of peptide signals were then compared among individuals between species and life stage/sex, among individuals used for growth inhibition assays, and between individuals that tested positive and negative for Bd. Only signals with strong intensities (>10% of the

total intensity measured) and mass to charge ratios within the range of the standards were included in the analysis. Signals within 2 Daltons were considered to be the same peptide, and those that were 22 (+/- 2) Daltons greater were considered to be the sodium adducts of the same peptide. Correlations between Bd inhibitory or enhancing assayed samples and the presence of common peptide signals (shared by two or more assayed samples) were determined using a one way ANOVA.

RESULTS

Amounts of peptides in skin secretion samples

Peptide amounts did not vary between *G. nebulanastes* and *G. excubitor* (Figure 2; df= 1, 77; p= 0.363), but amounts varied among life stages/sex (df=2,77 p<0.001) and due to an interactive effect between species and life stage/sex (df=1,77 p=0.013). Tukey's post hoc test indicated that the amounts of peptides recovered were lower in *G. nebulanastes* juveniles than in *G. excubitor* juveniles (df= 1,77 p= 0.011), *G. excubitor* females (df=1,77 p< 0.001), *G. excubitor* males (df= 1,77 p< 0.001), and G*. nebulanastes* males (df=1,77 p<0.001) (Figure 5). Within *G. excubitor* smaller peptide amounts were recovered from juveniles than from males (df= 2,43 p=0.012) or females (df=2,43 p=0.009).

Bd growth inhibition assays

Four out of the eight (50%) peptide samples from *G. nebulanastes* and five out of the nine (56%) samples from *G. excubitor* inhibited Bd (Figures 6-9). *Gastrotheca* species did not differ in their abilities to inhibit the growth of Bd (Figure 11, split-plot

ANOVA, df=1,15 p=0.96), but there was a difference in concentration (df=7,105 p=0.046). There was no interaction between species and peptide concentration (df=7,105 p=0.86). Inhibition was higher at 500 μg/mL than at 6.25 μg/mL (Tukey's post hoc test, df= 7,105 p=0.016).

Analysis via MALDI TOF mass spectrometry

The most common peptide signal shared between the two *Gastrotheca* species had a mass to charge ratio of 1430 +/- 2 Daltons; also present was its sodium adduct at 1452 +/- 2 Daltons. This signal occurred in 59% (27 of the 46) of the *G. excubitor* sampled, and in only one *G. nebulanastes*. Another frequent signal occurred at 1128 +/- 2 Daltons (Table 3). Interestingly, only *G. excubitor* expressed the peptide in this form (n=6), but both species expressed the sodium adduct at 1150 +/- 2 Daltons (four *G. excubitor* and nine *G. nebulanastes*). The frog species differed in the expression of several peptides. Most notably only *G. nebulanastes*, but 53% of sampled individuals, had a signal at 1688 +/- 2 Daltons. There was no correlation between peptide type and Bd growth inhibition or enhancement, except for a peptide at 1298 +/- 2 Daltons found in assayed *G. excubitor* samples. This peptide was correlated with Bd growth enhancement (ANOVA, df=1,7 p=0.011).

Prevalence and intensity of Bd infection

Five out of 36 *G. nebulanastes* (14%, CI=5%-26%) and eight out of 46 *G. excubitor* (17%, CI=8%-29%) tested positive for Bd. Infection levels varied from 0.14-125.66 zoospores in *G. nebulanastes* and from 0.04-1223.23 zoospores in *G. excubitor*. Of the assayed peptide samples, one came from an infected *G. nebulanastes* and one

came from an infected *G. excubitor*. This *G. nebulanastes* had a low infection level (ZE=0.69 zoospores) and its peptides exhibited Bd growth enhancement. The *G. excubitor* was the most infected individual (ZE=1223.23 zoospores) and its peptides were relatively strong Bd growth inhibitors (70% maximum Bd growth at 500 μg/mL) (Figure 8). There were no correlations between Bd infection and presence of peptide signals.

DISCUSSION:

The peptides we analyzed, from the Bd-susceptible *Gastrotheca nebulanastes* and the Bd-resistant *G. excubitor*, did not differ between species in quantity (Figure 5) or in their abilities to inhibit the growth of Bd (Figure 11). However, there was considerable variation among individuals, and some peptide samples exhibited patterns of growth inhibition while some exhibited growth enhancement (Figures 6-9).

Peptide profiles failed to completely explain variation among individuals because most peptide signals occurred in both inhibitory and enhancing peptide samples. Only the peptide at 1298 +/- 2 Daltons showed correlation with Bd growth enhancement in assayed *G. excubitor* samples. It is very likely that this peptide cannot inhibit Bd growth; and perhaps it is responsible for enhancing it by providing nourishment for zoospores. Although there were some shared peptide signals, overall each species produced unique peptide profiles suggesting interspecific variation that is consistent with previous research (Woodhams et al., 2007a, Rollins-Smith et al., 2006).

None of the assayed samples were able to completely inhibit Bd growth at any concentration. Therefore, no MIC values were generated to compare with peptides

analyzed by other studies. Interestingly, the peptide sample that demonstrated the most growth inhibition, allowing only 57% Bd growth at 500 μg/mL, came from the susceptible species, *G. nebulanastes* (Figure 6). Similarly, the susceptible *Rana muscosa* has been shown to have more potent anti-Bd AMPs than resistant congenerics (Woodhams et al., 2007b, Rollins-Smith et al., 2006). These results suggest that stronger peptides may be necessary to provide *in vivo* protection. However, even peptides capable of complete growth inhibition have been produced by susceptible frogs. This has been demonstrated in frogs from different families distributed worldwide, including: *Alytes obstetricans* in Europe (Bosch and Rincón, 2008), *Silurana tropicalis* in Africa (Parker et al., 2002), *Rana tarahuamara* in the United States (Rollins-Smith et al., 2002c), and *Hylomantis lemur* in South America (Conlon et al., 2007b).

It is difficult to attribute *G. excubitor's* chytridiomycosis resistance to AMPs because peptide samples from individuals within this species varied in their abilities to inhibit Bd growth: some peptide mixtures inhibited Bd growth, but some enhanced it (Figures 8 and 9). None of those that inhibited Bd growth were particularly strong (Figure 8). One of the strongest inhibiting peptide mixture was collected from the frog with the highest Bd load, suggesting that the Bd inhibition demonstrated by the assay might not translate to *in vivo* inhibition.

The prevalence of Bd reported from this study was low and similar for the *Gastrotheca* species. Historically, prevalence has been consistent for *G. nebulanastes* but has varied for *G. excubitor*, although overall values were relatively low. No individuals from either species tested positive for Bd in 2008 (Catenazzi et al., 2011). In 2009 prevalence was 25% and 18.2% (Catenazzi et al., 2011) and in 2012 prevalence

was 25% and 46.4% (Burkart et al., unpublished) for *G. nebulanastes* and *G. excubitor*, respectively. Despite the high prevalence for *G. excubitor* in 2012, individual infection levels were generally low for both species (Burkart et al., unpublished). The overall low prevalence of Bd infection observed between these species, despite their differences in susceptibilities, may be attributed to their similar life history characteristics. Because they are direct-developers (Duellman and Maness, 1980), it is unlikely that either utilizes bodies of water, where Bd infection typically occurs. This may also explain why *G. nebulanastes* coexists with Bd despite being susceptible to chytridiomycosis.

Perhaps the difference in susceptibility between these frogs can be attributed to another defense mechanism such as anti-Bd skin bacteria. This defense has been associated with chytridiomycosis resistance (Woodhams et al., 2007b, Becker and Harris, 2010, Park et al., 2014). In a concurrent study we found that *G. excubitor* has more and stronger anti-Bd skin bacteria than *G. nebulanastes*. Therefore, it is likely that bacteria plays a more important role in protecting *G. excubitor* from Bd than AMPs.

CONCLUSIONS:

Understanding what protects resistant frog species from chytridiomycosis can assist in the development of mitigation strategies. Our results suggest that, at least for these marsupial frogs, peptides are not important contributors to chytridiomycosis resistance. Perhaps other defenses like cutaneous bacteria are. So far there has been some progress made towards developing a bioaugmentation strategy using probiotic bacteria to protect susceptible frogs. Although this method is still in development, it has been implemented successfully *in vitro*, and there are currently no proposals to use

AMPs as a mitigation strategy. However, it will be important to continue exploring the anti-Bd abilities of both of these defenses. Both skin bacteria and AMPs coexist in the microbiome of the skin, and it is likely that interactions between the two dictate their anti-Bd abilities *in vivo*.

Table 2: Solutions used for AMP collection.

Solution	Purpose	Ingredients	Reference
Norepinephrine Injection (40 nmol norepinephrine)	Stimulate the release of AMPs	Norepinephrine, APBS	Rollins-Smith et al. 2005
Amphibian Phosphate Buffered Saline (APBS)	Solvent for norepinephrine	6.6 g of NaCl, 1.15 g of anhydrous Na2HPO4, 0.2 g of KH2PO4, per 1 liter of ultra-pure (HPLC grade) water	Doug Woodhams, pers. comm.
50% HCl buffer	Acidify skin washings	550 ml of concentrated HCl, 500 ml HPLC water	Doug Woodhams, pers. comm.
Buffer A (0.1% HCl)	Activating Sep Pak cartridges	1 ml of concentrated HCl, 1 liter of HPLC water	Doug Woodhams, pers. comm.
Buffer B (70% acetonitrile)	Elution of peptides from Sep Pak cartridges	700 ml of acetonitrile, 1 ml of concentrated HCl, and 300 ml of HPLC grade water	Louise Rollins Smith, pers. comm.

Table 3: Numbers of *Gastrotheca* individuals with common peptide signals. The top row is *G. nebulanastes* and the bottom *G. excubitor*.

	Peptide m/z									
1128/1150	**1226**	**1298**	**1430/1452**	**1437**	**1688**	**1911**	**2160**	**2164**	**3164**	**3742**
6/4	0	6	22/5	0	0	5	5	4	0	5
0/9	9	3	1/0	5	19	0	0	0	7	0

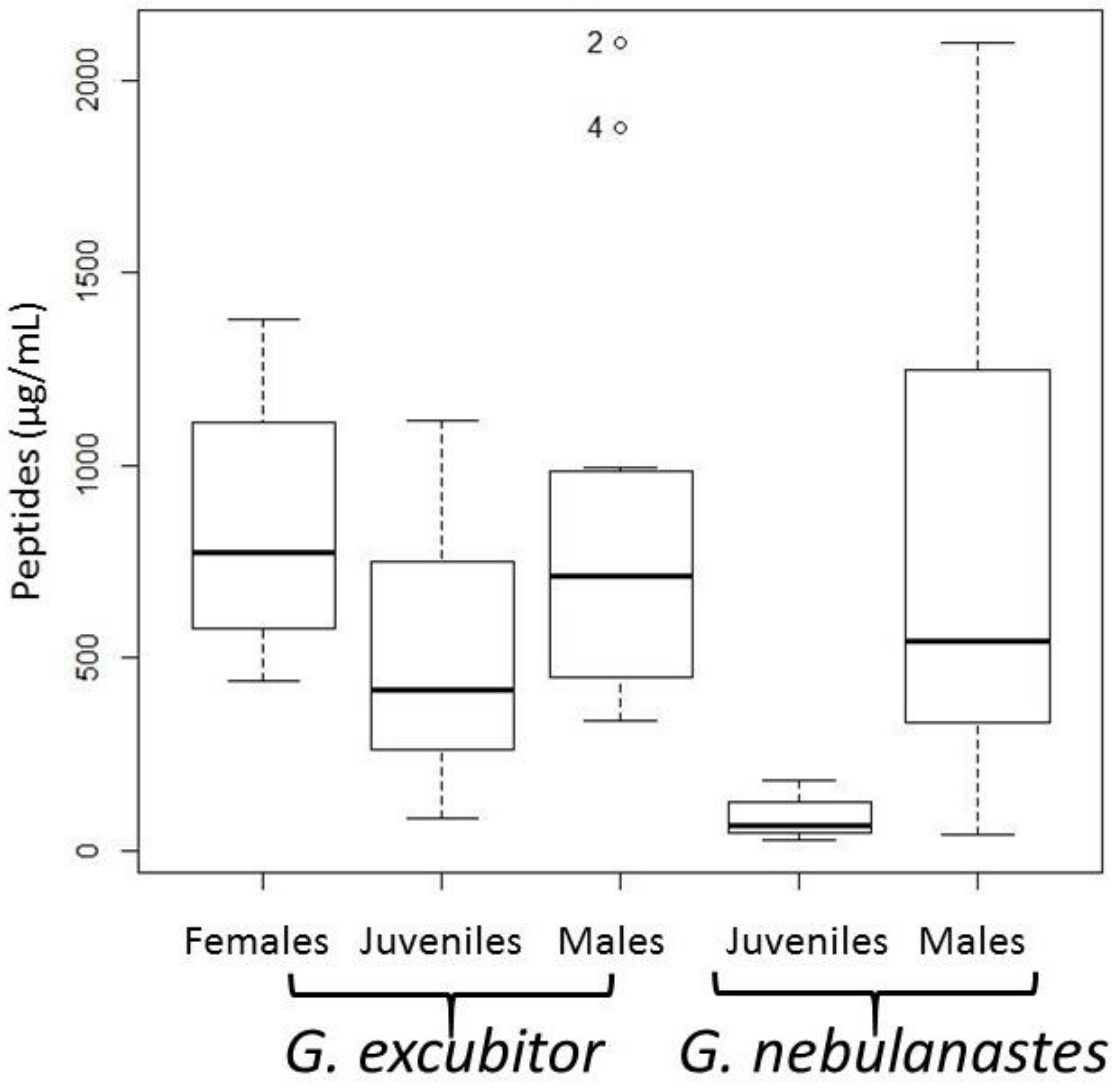

Figure 5: Mean, quartiles, and range of peptide mixture amounts recovered for all individuals (dots indicate outliers).

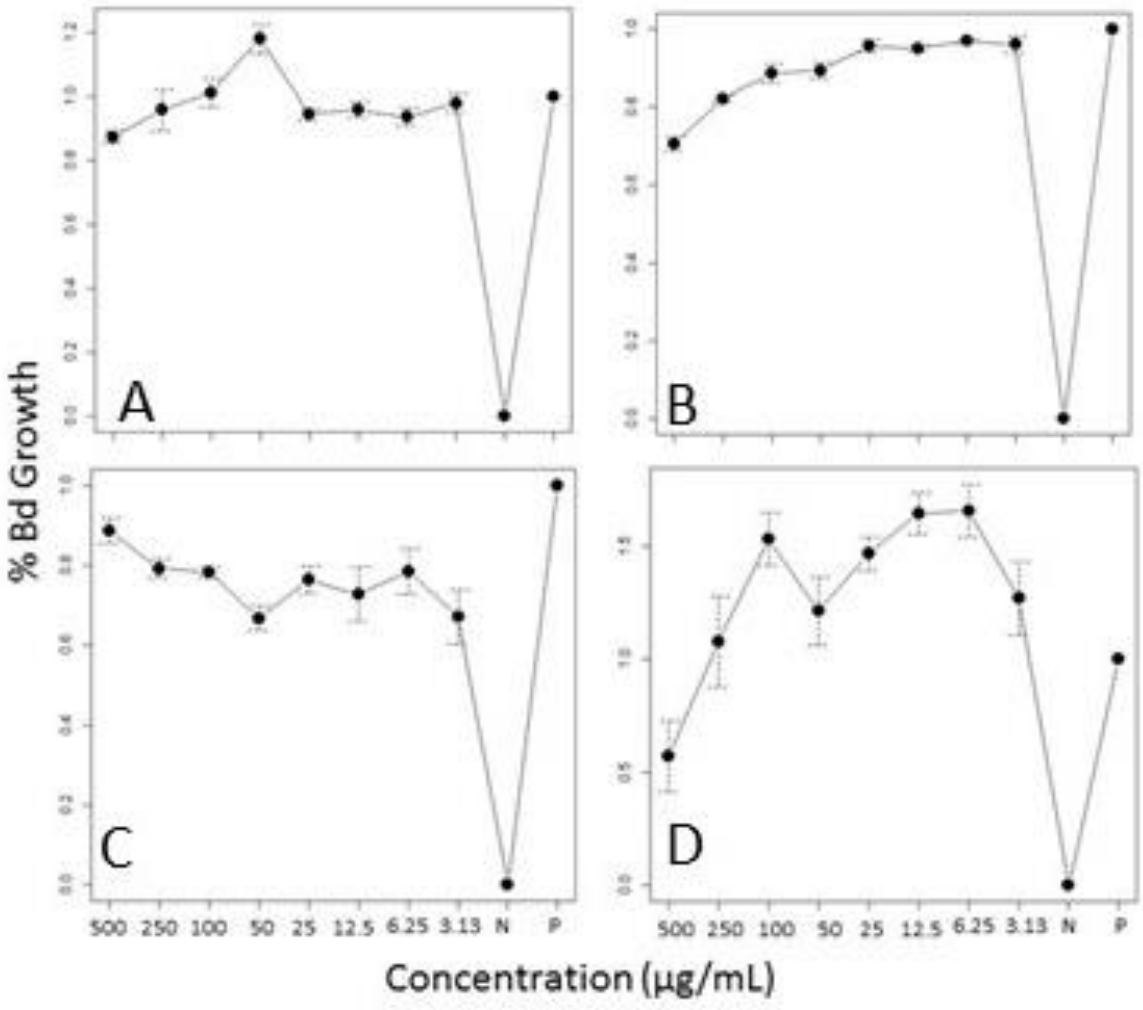

Figure 6: Mean ± SE change in absorbance, pA_{490}, as a function of peptide mixture concentration (500-3.125 µg/mL), for peptide mixtures of *G. nebulanastes* that inhibited Bd. N indicates negative control and P positive control. Each panel (A-D) represents the results from an indivual peptide sample.

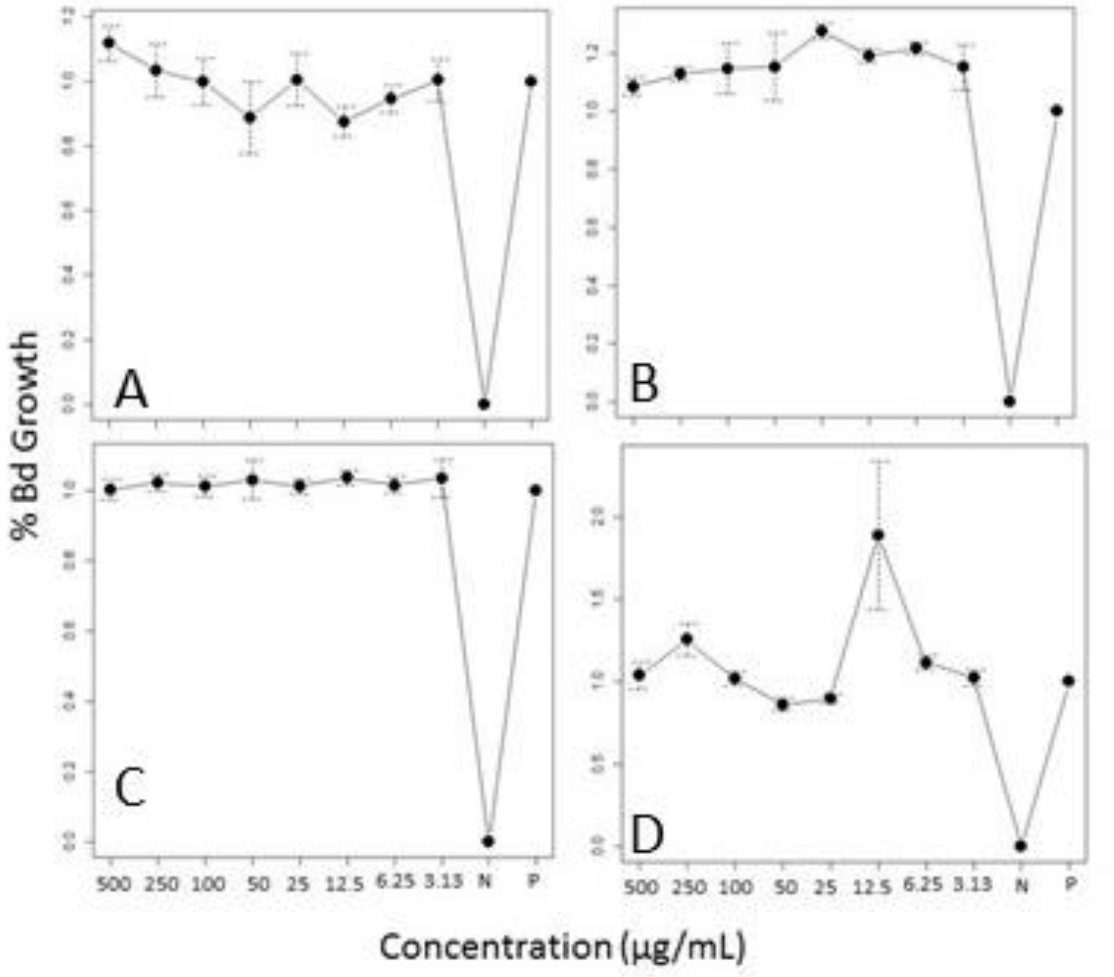

Figure 7: Change in absorbance, pA_{490} (mean ± SE), as a function of peptide mixture concentration (500-3.125 µg/mL), for samples from *G. nebulanastes* that did not inhibit Bd. N indicates negative control and P positive control. Each panel (A-D) represents the result from an individual peptide sample.

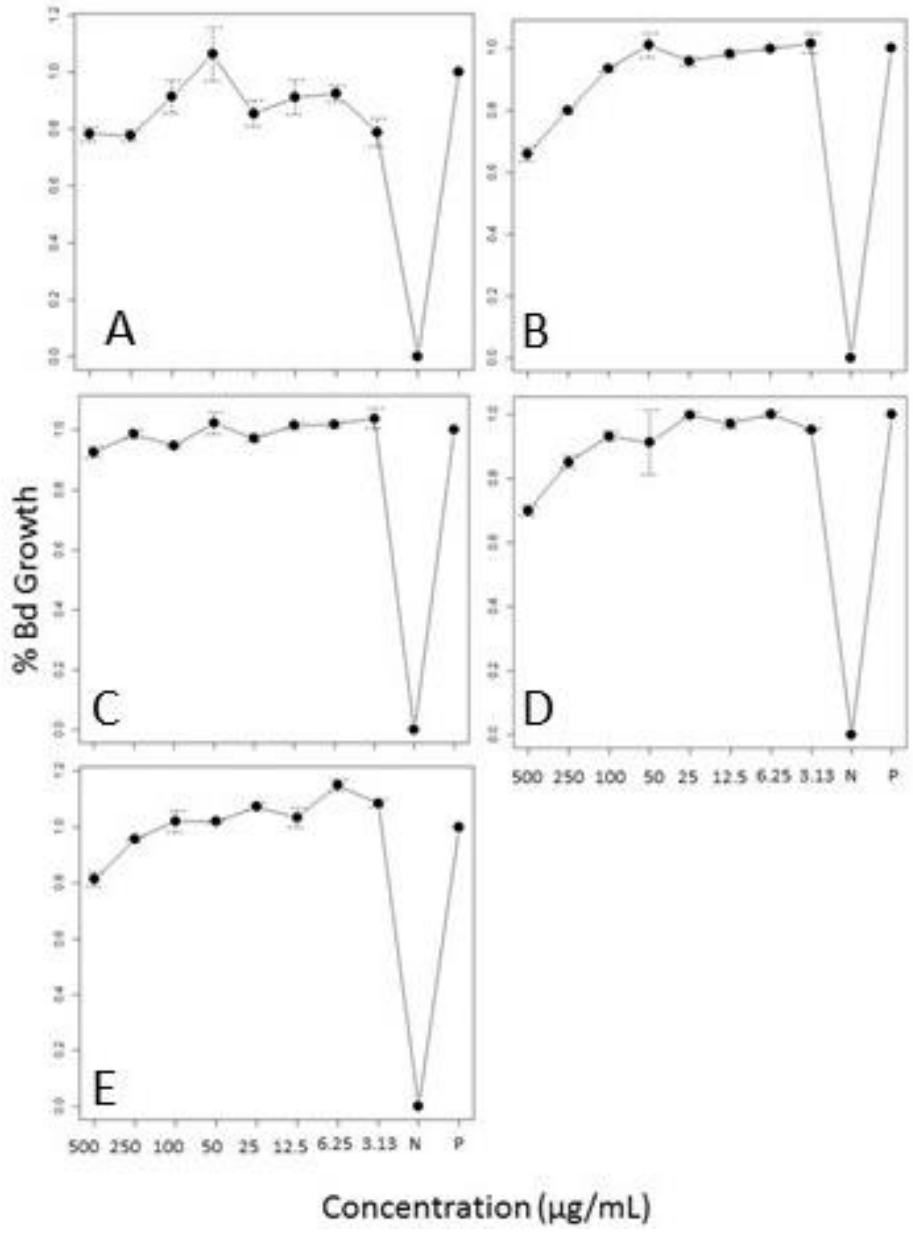

Figure 8: Mean ± SE change in absorbance, pA_{490}, as a function of peptide mixture concentration (500-3.125 µg/mL), for peptide mixtures of *G. excubitor* that inhibited Bd. N indicates negative control and P positive control. Each panel (A-E) represents the results from an indivual peptide sample.

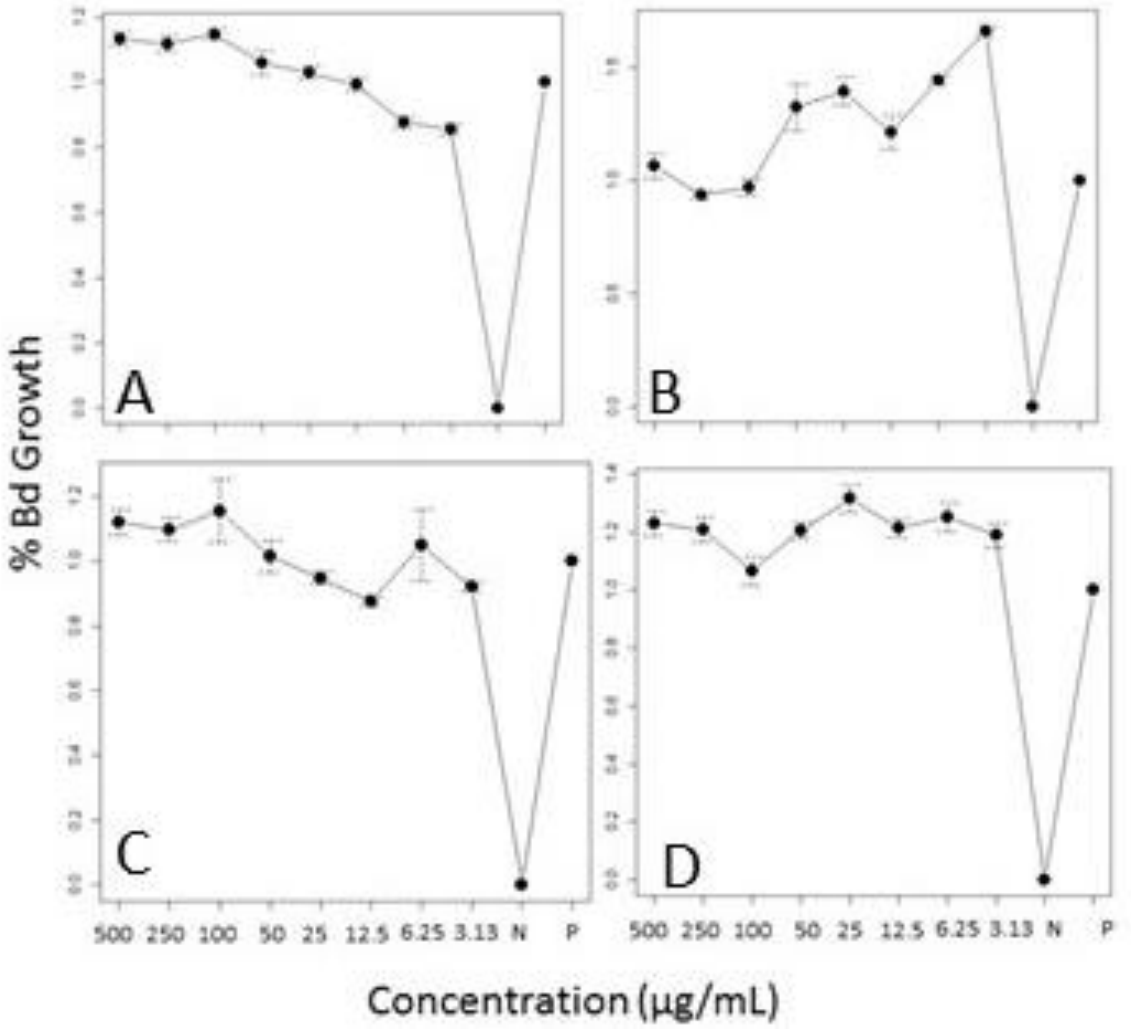

Figure 9: Mean ± SE change in absorbance, pA_{490}, as a function of peptide mixture concentration (500-3.125 µg/mL), for peptide mixtures of *G. excubitor* that did not inhibit Bd. N indicates negative control and P positive control. Each panel (A-D) represents the results from an indivual peptide sample.

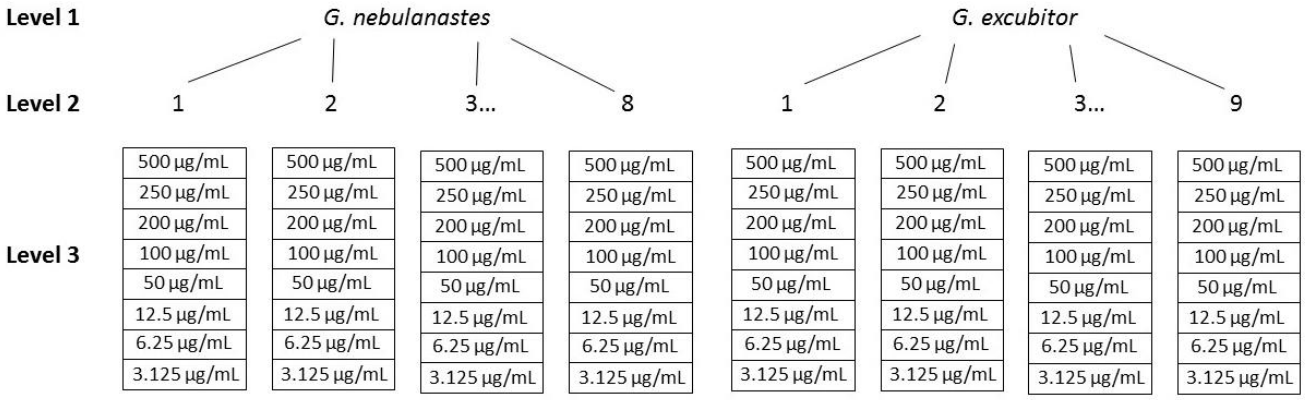

Figure 10: The split plot design of the Bd growth inhibition assays. Level 1 is species, Level 2 individuals sampled (n=8 for *G. nebulanastes* and n=9 for *G. excubitor*), and Level 3 are the different peptide concentrations tested. There were five replicates of every concentration for each individual, but replicates were average for analysis.

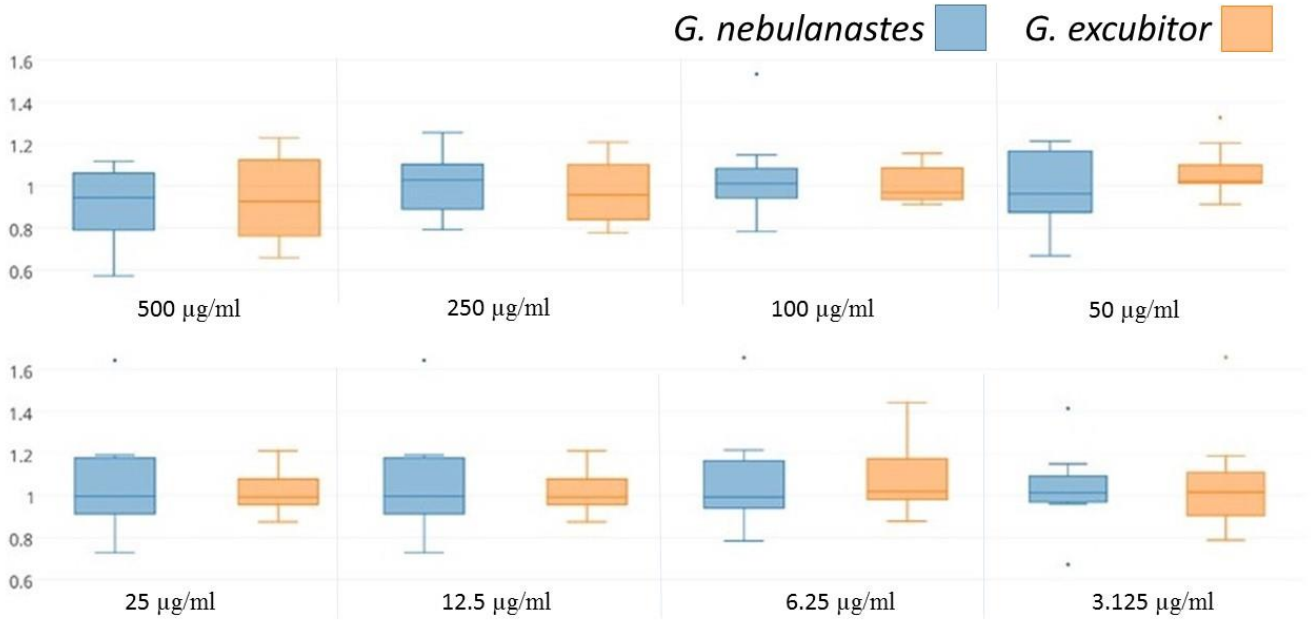

Figure 11: Growth for each sample assayed (mean of the five replicates) per peptide concentration. Blue indicates values for *G. nebulanastes* and orange values for *G. excubitor*.

REFERENCES

Abramoff, M. D., Magalhaes, P. J. & Ram, S. J. Image processing with ImageJ. *Biophotonics International***,** 36-43.

Amiche, M., Seon, A. A., Pierre, T. N. & Nicolas, P. 1999. The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. *Febs Letters,* 456**,** 352-356.

Antwis, R. E., Haworth, R. L., Engelmoer, D. J. P., Ogilvy, V., Fidgett, A. L. & Preziosi, R. F. 2014. Ex situ Diet influences the bacterial community associated with the skin of Red-Eyed Tree Frogs (*Agalychnis callidryas*). *Plos One,* 9.

Apponyi, M. A., Pukala, T. L., Brinkworth, C. S., Maselli, V. M., Bowie, J. H., Tyler, M. J., Booker, G. W., Wallace, J. C., Carver, J. A., Separovic, F., Doyle, J. & Llewellyn, L. E. 2004. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides,* 25**,** 1035-1054.

Bandounas, L., Wierckx, N. J., de Winde, J. H. & Ruijssenaars, H. J. 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotechnology,* 11**,** 94-94.

Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N. & Minbiole, K. P. C. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Applied and Environmental Microbiology,* 75**,** 6635-6638.

Becker, M. H. & Harris, R. N. 2010. Cutaneous bacteria of the Redback Salamander prevent morbidity associated with a lethal disease. *Plos One,* 5.

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., Brucker, R. M., Domangue, R. J. & Gratwicke, B. 2011. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian Golden Frogs. *Ecohealth,* 8**,** 501-506.

Belden, L. K. & Harris, R. N. 2007. Infectious diseases in wildlife: The community ecology context. Ecological Society of America.

Bell, S. C., Alford, R. A., Garland, S., Padilla, G. & Thomas, A. D. 2013. Screening bacterial metabolites for inhibitory effects against Batrachochytrium dendrobatidis using a spectrophotometric assay. *Diseases of Aquatic Organisms,* 103**,** 77-+.

Benson, B. J. & Hadley, M. E. 1969. In vitro characterization of adrenergic receptors controlling skin gland secretion in two anurans Rana pipiens and Xenopus laevis. *Comparative Biochemistry And Physiology,* 30**,** 857,IN7,863-862,IN7,864.

Bergaoui, I., Zairi, A., Gharsallah, H., Aouni, M., Hammami, A., Hani, K. & Selmi, B. 2013. The in vitro evaluation of anti-chlamydial and cytotoxic properties of dermaseptin S-4 and derivatives: peptides from amphibian skin. *Medicinal Chemistry Research,* 22**,** 6096-6104.

Berger, L., Hyatt, A. D., Speare, R. & Longcore, J. E. 2005a. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 68**,** 51-63.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. & Parkes, H. 1998a. Chytridiomycosis causes amphibian

mortality associated with population declines in the rain. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9031.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. & Parkes, H. 1998b. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9031-9036.

Berger, L., Speare, R. & Skerratt, L. F. 2005b. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs Litoria caerulea with severe chytridiomycosis. *Diseases of Aquatic Organisms,* 68**,** 65-70.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C. & Harris, R. N. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters,* 16**,** 807-820.

Bosch, J. & Rincón, P. A. 2008. Chytridiomycosis-mediated expansion of Bufo bufo in a montane area of Central Spain: an indirect effect of the disease. *Diversity & Distributions,* 14**,** 637-643.

Bovbjerg, A. M. 1963. Development of glands of dermal plicae in *Rana pipiens*. *Journal of Morphology,* 113**,** 231-&.

Bowerman, J., Rombough, C., Weinstock, S. R. & Padgett-Flohr, G. E. 2010. Terbinafine hydrochloride in ethanol effectively clears *Batrachochytrium*

dendrobatidis in amphibians. *Journal of Herpetological Medicine & Surgery,* 20**,** 24-28.

Bowie, J. H., Wegener, K. L., Chia, B. C. S., Wabnitz, P. A., Carver, J. A., Tyler, M. J. & Wallace, J. C. 1999. Host defence antibacterial peptides from skin secretions of Australian amphibians. The relationship between structure and activity. *Protein and Peptide Letters,* 6**,** 259-269.

Bresciano, J., Salvador, C., Paz-y-Miño, C., Parody-Merino, A., Bosch, J. & Woodhams, D. 2015. Variation in the presence of anti- *Batrachochytrium dendrobatidis* bacteria of amphibians across life stages and elevations in Ecuador. *EcoHealth,* 12**,** 310-319.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. & Rachowicz, L. J. 2005. Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology,* 86**,** 3149-3159.

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A. & Minbiole, K. P. C. 2008. Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology,* 34**,** 1422-1429.

Carey, C. & Alexander, M. A. 2003. Climate Change and Amphibian Declines: Is There a Link? Blackwell Science.

Catenazzi, A., Lehr, E., Rodriguez, L. O. & Vredenburg, V. T. 2011. *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. *Conservation Biology,* 25**,** 382-391.

Chatfield, M. W. H. & Richards-Zawacki, C. L. 2011. Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of Aquatic Organisms,* 94**,** 235-238.

Conlon, J. M. 2011. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. *Cell and Tissue Research,* 343**,** 201-212.

Conlon, J. M., Al-Dhaheri, A., Al-Mutawa, E., Al-Kharrge, R., Ahmed, E., Kolodziejek, J., Nowotny, N., Nielsen, P. F. & Davidson, C. 2007a. Peptide defenses of the Cascades frog *Rana cascadae*: implications for the evolutionary history of frogs of the Amerana species group. *Peptides,* 28**,** 1268-1274.

Conlon, J. M., Kolodziejek, J. & Nowotny, N. 2004a. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1696**,** 1-14.

Conlon, J. M., Kolodziejek, J. & Nowotny, N. 2004b. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1696**,** 1-14.

Conlon, J. M., Reinert, L. K., Mechkarska, M., Prajeep, M., Meetani, M. A., Coquet, L., Jouenne, T., Hayes, M. P., Padgett-Flohr, G. & Rollins-Smith, L. A. 2013. Evaluation of the skin peptide defenses of the Oregon Spotted Frog *Rana pretiosa* against infection by the chytrid fungus *Batrachochytrium dendrobatidis. Journal of Chemical Ecology,* 39**,** 797-805.

Conlon, J. M., Woodhams, D. C., Raza, H., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H. & Rollins-Smith, L. A. 2007b. Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (Hylidae : Phyllomedusinae). *Toxicon,* 50**,** 498-506.

Daszak, P. & Berger, L. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases,* 5**,** 735.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. 2003. Infectious disease and amphibian population declines. Blackwell Science.

Davidson, C., Shaffer, H. B. & Jennings, M. R. 2001. Declines of the California red-legged frog: Climate, UV-B, habitat, and pesticides hypotheses. *Ecological Applications,* 11**,** 464-479.

del Pino, E. M. & Escobar, B. 1981. Embryonic stages of Gastrotheca riobambae (Fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. *Journal Of Morphology,* 167**,** 277-295.

Dockray, G. J. & Hopkins, C. R. 1975. Caerulein secretion by dermal glands in *Xenopus laevis*. *Journal of Cell Biology,* 64**,** 724-733.

Duellman, W. E., Catenazzi, A. & Blackburn, D. C. 2011. A new species of marsupial frog (Anura: Hemiphractidae: Gastrotheca) from the Andes of southern Peru. *Zootaxa***,** 1-14.

Duellman, W. E. & Maness, S. J. 1980. The reproductive behavious of some Huylid marsupial frogs. *Journal of Herpetology,* 14**,** 213-222.

Fierer, N., Ferrenberg, S., Flores, G. E., Gonzalez, A., Kueneman, J., Legg, T., Lynch, R. C., McDonald, D., Mihaljevic, J. R., O'Neill, S. P., Rhodes, M. E., Song, S. J. &

Walters, W. A. 2012. From animalcules to an ecosystem: Application of ecological concepts to the human microbiome. *Annual Review of Ecology, Evolution, and Systematics, Vol 43,* 43**,** 137-155.

Fisher, M. C., Garner, T. W. J. & Walker, S. F. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology,* 63**,** 291-310.

Fisher, R. N. & Shaffer, H. B. 1996. The decline of amphibians in California's great central valley. Blackwell Scientific Publications.

Fites, J. S., Parker, C. S. M., Oswald-Richter, K. A., Ramsey, J. R., Gammill, W. M. & Rollins-Smith, L. A. 2013a. *Batrachochytrium dendrobatidis*, an emergent pathogen linked to amphibian declines, produces factors that inhibit adaptive immunity in amphibians and mammals. *Integrative and Comparative Biology,* 53**,** E69-E69.

Fites, J. S., Ramsey, J. P., Holden, W. M., Collier, S. P., Sutherland, D. M., Reinert, L. K., Gayek, A. S., Dermody, T. S., Aune, T. M., Oswald-Richter, K. & Rollins-Smith, L. A. 2013b. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science,* 342**,** 366-369.

Fites, J. S., Ramsey, J. P. & Rollins-Smith, L. A. 2012. Paralysis of amphibian lymphocyte functions by products of the chytrid fungus, *Batrachocytrium dendrobatidis*. *Integrative and Comparative Biology,* 52**,** E58-E58.

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S. & Amezquita, A. 2012. Surviving chytridiomycosis: Differential anti-

Batrachochytrium dendrobatidis activity in bacterial isolates from three lowland species of Atelopus. *Plos One,* 7.

Gammill, W. M., Fites, J. S. & Rollins-Smith, L. A. 2012. Norepinephrine depletion of antimicrobial peptides from the skin glands of *Xenopus laevis*. *Developmental and Comparative Immunology,* 37**,** 19-27.

Garner, T. W. J., Garcia, G., Carroll, B. & Fisher, M. C. 2009. Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of Alytes muletensis tadpoles. *DISEASES OF AQUATIC ORGANISMS,* 83**,** 257-260.

Geiger, C. C., Küpfer, E., Schär, S., Wolf, S. & Schmidt, B. R. 2011. Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*. *Amphibia-Reptilia,* 32**,** 276-280.

Godfrey, S. A., Harrow, S. A., Marshall, J. W. & Klena, J. D. 2001. Characterization by 16S rRNA sequence analysis of pseudomonads causing blotch disease of cultivated *Agaricus bisporus*. *Applied And Environmental Microbiology,* 67**,** 4316-4323.

Goraya, J., Knoop, F. C. & Conlon, J. M. 1998. Ranatuerins: Antimicrobial peptides isolated from the skin of the American bullfrog, *Rana catesbeiana*. *Biochemical and Biophysical Research Communications,* 250**,** 589-592

Haas, D. & Defago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology,* 3**,** 307-319.

Hadfield, C. A. & Whitaker, B. R. 2005. Amphibian emergency medicine and care. *Seminars in Avian and Exotic Pet Medicine,* 14**,** 79-89.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T. & Minbiole, K. P. C. 2009a. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *Isme Journal,* 3**,** 818-824.

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A. & Patel, A. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of Amphibian species. *Ecohealth,* 3**,** 53-56.

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L. & Alford, R. A. 2009b. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Diseases of Aquatic Organisms,* 83**,** 11-16.

Hill, T. C. J., Moffett, B. F., DeMott, P. J., Georgakopoulos, D. G., Stump, W. L. & Franc, G. D. 2014. Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR. *Applied & Environmental Microbiology,* 80**,** 1256-1267.

Holden, W. M., Reinert, L. K., Hanlon, S. M., Parris, M. J. & Rollins-Smith, L. A. 2015. Development of antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*. *Developmental and Comparative Immunology,* 48**,** 65-75.

Holmes, C. & Balls, M. 1978. In vitro studies on the control of myoepithelial cell contraction in the granular glands of *Xenopus laevis* skin. *General and Comparative Endocrinology,* 36**,** 255-263.

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason,

F. & Colling, A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 73**,** 175-192.

Jani, A. J. & Briggs, C. J. 2014. The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** E5049-E5058.

Jones, M. E. B., Paddock, D., Bender, L., Allen, J. L., Schrenzel, M. S. & Pessier, A. P. 2012. Treatment of chytridiomycosis with reduced-dose itraconazole. *Diseases of Aquatic Organisms,* 99**,** 243-249.

Kiesecker, J. M., Blaustein, A. R. & Belden, L. K. 2001. Complex causes of amphibian population declines. (cover story). *Nature,* 410**,** 681.

Kilpatrick, A. M., Briggs, C. J. & Daszak, P. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution,* 25**,** 109-118.

Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R. & Otvos, L. 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *BIOCHEMISTRY,* 40**,** 3016-3026.

König, E., Bininda-Emonds, O. R. P. & Shaw, C. 2015. Review: The diversity and evolution of anuran skin peptides. *Peptides,* 63**,** 96-117.

Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E. & Woodhams, D. C. 2014. Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS ONE,* 9**,** e87101-e87101.

Lam, B. A., Walke, J. B., Vredenburg, V. T. & Harris, R. N. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation,* 143**,** 529-531.

Lam, B. A., Walton, D. B. & Harris, R. N. 2011. Motile Zoospores of *Batrachochytrium dendrobatidis* move away from antifungal metabolites produced by amphibian skin bacteria. *Ecohealth,* 8**,** 36-45.

Lam, B. A., Woodhams, D. C., Vredenburg, V. T. & Harris, R. N. 2008. Survey and experimental evidence that symbiotic antifungal bacteria protect frog populations from a lethal skin pathogen. *Abstracts of the General Meeting of the American Society for Microbiology,* 108**,** 450-450.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. & Collins, J. P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3165-3170.

Lips, K. R., Diffendorfer, J., Mendelson, J. R., III & Sears, M. W. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. *Plos Biology,* 6**,** 441-454.

Longcore, J. E., Pessier, A. P. & Nichols, D. K. 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia,* 91**,** 219-227.

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V. & Harris, R. N. 2014. Microbial community dynamics and effect of environmental

microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *Isme Journal,* 8**,** 830-840.

Marsh, D. M. & Trenham, P. C. 2001. Metapopulation Dynamics and Amphibian Conservation. Blackwell Science.

Martel, A., Van Rooij, P., Vercauteren, G., Baert, K., Van Waeyenberghe, L., Debacker, P., Garner, T. W. J., Woeltjes, T., Ducatelle, R., Haesebrouck, F. & Pasmans, F. 2011. Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *MEDICAL MYCOLOGY,* 49**,** 143-149.

Matsuzaki, K. 1999. Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *BIOCHIMICA ET BIOPHYSICA ACTA-BIOMEMBRANES,* 1462**,** 1-10.

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R. & Lauber, C. L. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *Isme Journal,* 6**,** 588-596.

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., Halstead, N. T., Lentz, G., Tenouri, N., Young, S., Civitello, D. J., Ortega, N., Fites, J. S., Reinert, L. K., Rollins-Smith, L. A., Raffel, T. R. & Rohr, J. R. 2014. Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature,* 511**,** 224-+.

Mechkarska, M., Attoub, S., Sulaiman, S., Pantic, J., Lukic, M. L. & Michael Conlon, J. 2014. Anti-cancer, immunoregulatory, and antimicrobial activities of the frog skin

host-defense peptides pseudhymenochirin-1Pb and pseudhymenochirin-2Pa. *Regulatory Peptides,* 194-195**,** 69-76.

Mills, J. W. & Prum, B. E. 1984. Morphology of the exocrine glands of the frog skin. *American Journal of Anatomy,* 171**,** 91.

Muletz, C. R., Myers, J. M., Domangue, R. J., Herrick, J. B. & Harris, R. N. 2012. Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biological Conservation,* 152**,** 119-126.

Myers, J. M., Ramsey, J. P., Blackman, A. L., Nichols, A. E., Minbiole, K. P. C. & Harris, R. N. 2012. Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: The combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa*. *Journal of Chemical Ecology,* 38**,** 958-965.

Müller, H., Zachow, C., Alavi, M., Tilcher, R., Krempl, P. M., Thallinger, G. G. & Berg, G. 2013. Complete genome sequence of the sugar beet endophyte *Pseudomonas poae* RE*1-1-14, a Disease-Suppressive Bacterium. *Genome Announcements,* 1**,** e0002013-e0002013.

Nicolas, P. & Mor, A. 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annual Review of Microbiology,* 49**,** 277.

Nutkins, J. C. & Williams, D. H. 1989. Identification of highly acidic peptides from processing of the skin prepropeptides of *Xenopus laevis*. *European Journal of Biochemistry,* 181**,** 97-102.

Park, S. T., Collingwood, A. M., St-Hilaire, S. & Sheridan, P. P. 2014. Inhibition of *Batrachochytrium dendrobatidis* caused by bacteria isolated from the skin of Boreal Toads, *Anaxyrus (Bufo) boreas boreas*, from Grand Teton National Park, Wyoming, USA. *Microbiology insights,* 7**,** 1-8.

Parker, J. M., Mikaelian, I., Hahn, N. & Diggs, H. E. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comparative Medicine,* 52**,** 265-268.

Pask, J. D., Woodhams, D. C. & Rollins-Smith, L. A. 2012. The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. *Global Change Biology,* 18**,** 1231-1238.

Pasupuleti, M., Schmidtchen, A. & Malmsten, M. 2012. Antimicrobial peptides: key components of the innate immune system. *Critical Reviews in Biotechnology,* 32**,** 143-171.

Pessier, A. P., Nichols, D. K., Longcore, J. E. & Fuller, M. S. 1999. Cutaneous chytridiomycosis in poison dart frogs (Dendrobates spp.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation,* 11**,** 194-199.

Pounds, J. A., Fogden, M. P. L. & Campbell, J. H. 1999. Biological response to climate change on a tropical mountain. *Nature,* 398**,** 611.

Pounds, J. A., Fogden, M. P. L., Savage, J. M. & Gorman, G. C. 1997. Tests of null models for amphibian declines on a tropical mountain. *Conservation Biology,* 11**,** 1307-1322.

Rachowicz, L. J., Knapp, R. A., Morgan, J. A. T., Stice, M. J., Vredenburg, V. T., Parker, J. M. & Briggs, C. J. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology,* 87**,** 1671-1683.

Ramsey, J. P., Reinert, L. K., Harper, L. K., Woodhams, D. C. & Rollins-Smith, L. A. 2010. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African Clawed Frog, *Xenopus laevis*. *Infection and Immunity,* 78**,** 3981-3992.

Resnick, N. M., Maloy, W. L., Guy, H. R. & Zasloff, M. 1991. A novel endopeptidase from Xenopus that recognizes alpha-helical secondary structure. *Cell,* 66**,** 541-554.

Rinaldi, A. C. 2002. Antimicrobial peptides from amphibian skin: an expanding scenario - Commentary. *CURRENT OPINION IN CHEMICAL BIOLOGY,* 6**,** 799-804.

Robertson, L. S., Fellers, G. M., Marranca, J. M. & Kleeman, P. M. 2013. Expression analysis and identification of antimicrobial peptide transcripts from six North American frog species. *Diseases of Aquatic Organisms,* 104**,** 225-+.

Rollins-Smith, L., Woodhams, D., Reinert, L., Vredenburg, V., Briggs, C., Nielsen, P. & Conlon, J. 2006. Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental and Comparative Immunology,* 30**,** 831-842.

Rollins-Smith, L. A., Carey, C., Longcore, J., Doersam, J. K., Boutte, A., Bruzgal, J. E. & Conlon, J. M. 2002a. Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Developmental and Comparative Immunology,* 26**,** 471-479.

Rollins-Smith, L. A., Doersam, J. K., Longcore, J. E., Taylor, S. K., Shamblin, J. C., Carey, C. & Zasloff, M. A. 2002b. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Developmental and Comparative Immunology,* 26**,** 63-72.

Rollins-Smith, L. A., Ramsey, J. P., Reinert, L. K. & Woodhams, D. C. 2011. Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology,* 51**,** E117-E117.

Rollins-Smith, L. A., Reinert, L. K., Miera, V. & Conlon, J. M. 2002c. Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. *Biochemical and Biophysical Research Communications,* 297**,** 361-367.

Rollins-Smith, L. A., Reinert, L. K., O'Leary, C. J., Houston, L. E. & Woodhams, D. C. 2005. Antimicrobial peptide defenses in amphibian skin. *Integrative and Comparative Biology,* 45**,** 137-142.

Roth, T., Foley, J., Worth, J., Piovia-Scott, J., Pope, K. & Lawler, S. 2013. Bacterial flora on Cascades frogs in the Klamath mountains of California. *Comparative Immunology, Microbiology and Infectious Diseases,* 36**,** 591-598.

Scheuring, I., Yu, D. W. & Baalen, M. 2012. How to assemble a beneficial microbiome in three easy steps. *Ecology Letters,* 15**,** 1300-1307.

Schumacher, U., Adam, E., Hauser, F., Probst, J. C. & Hoffmann, W. 1994. Molecular anatomy of a skin gland: histochemical and biochemical investigations on the mucous glands of *Xenopus laevis*. *The Journal Of Histochemistry And Cytochemistry: Official Journal Of The Histochemistry Society,* 42**,** 57-65.

Simmaco, M., Mignogna, G. & Barra, D. 1998. Antimicrobial peptides from amphibian skin: What do they tell us? *Biopolymers,* 47**,** 435-450.

Sjöberg, E. & Flock, A. 1976. Innervation of skin glands in the frog. *Cell And Tissue Research,* 172**,** 81-91.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B. & Kenyon, N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *ECOHEALTH,* 4**,** 125-134.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry,* 150**,** 76-85.

Steinborner, S. T., Waugh, R. J., Bowie, J. H., Wallace, J. C., Tyler, M. J. & Ramsay, S. L. 1997. New caerin antibacterial peptides from the skin glands of the Australian tree frog *Litoria xanthomera*. *Journal Of Peptide Science: An Official Publication Of The European Peptide Society,* 3**,** 181-185.

Stice, M. J. & Briggs, C. J. 2010. Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Journal of Wildlife Diseases,* 46**,** 70-77.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Ana, S. L. R., Fischman, D. L. & Waller, R. W. 2004. Status and trends of amphibian declines and extinctions worldwide. American Association for the Advancement of Science.

Tobler, U. & Schmidt, B. R. 2010. Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE,* 5**,** 1-8.

Tyler, M. J., Stone, D. J. M. & Bowie, J. H. 1992. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *Journal of Pharmacological and Toxicological Methods,* 28**,** 199-200.

VanCompernolle, S. E., Taylor, R. J., Oswald-Richter, K., Jiang, J. Y., Youree, B. E., Bowie, J. H., Tyler, M. J., Conlon, J. M., Wade, D., Aiken, C., Dermody, T. S., KewalRamani, V. N., Rollins-Smith, L. A. & Unutmaz, D. 2005. Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. *Journal of Virology,* 79**,** 11598-11606.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. American Association for the Advancement of Science.

Vredenburg, V. T., Briggs, C. J. & Harris, R. 2011. Host-pathogen dynamics of amphibian chytridiomycosis: The role of the skin microbiome in health and disease. In: Olsen, L. (ed.). Washington DC, National Academies Press.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. & Briggs, C. J. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 9689-9694.

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A. & Woodhams, D. C. 2011. Social immunity in amphibians: Evidence for vertical transmission of innate defenses. *Biotropica,* 43**,** 396-400.

Westerhoff, H. V., Juretić, D., Hendler, R. W. & Zasloff, M. 1989. Magainins and the disruption of membrane-linked free-energy transduction. National Academy of Sciences of the United States of America.

Winder, R. S., Macey, D. E. & Cortese, J. 2010. Dominant bacteria associated with broods of mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae). *Journal of the Entomological Society of British Columbia,* 107**,** 43-56.

Woodhams, D., Kenyon, N., Bell, S., Alford, R., Chen, S., Billheimer, D., Shyr, Y. & Rollins-Smith, L. 2010a. Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata*. *Diversity and Distributions,* 16**,** 703-712.

Woodhams, D., Voyles, J., Lips, K., Carey, C. & Rollins-Smith, L. 2006a. Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases,* 42**,** 207-218.

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., Bell, S. C., Bletz, M., Daskin, J. H., Davis, L. R., Flechas, S. V., Lauer, A., Gonzalez, A., Harris, R. N., Holden, W. M., Hughey, M. C., IbÁÑEz, R., Knight, R. O. B., Kueneman, J. & Rabemananjara, F. 2015. Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology,* 96**,** 595-595.

Woodhams, D. C., Alford, R. A. & Marantelli, G. 2003. Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms,* 55**,** 65-67.

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K. & Rollins-Smith, L. A. 2007a. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation,* 10**,** 409-417.

Woodhams, D. C., Bigler, L. & Marschang, R. 2012a. Tolerance of fungal infection in European water frogs exposed to *Batrachochytrium dendrobatidis* after experimental reduction of innate immune defenses. *Bmc Veterinary Research,* 8.

Woodhams, D. C., Geiger, C. C., Reinert, L. K., Rollins-Smith, L. A., Lam, B., Harris, R. N., Briggs, C. J., Vredenburg, V. T. & Voyles, J. 2012b. Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Diseases of Aquatic Organisms,* 98**,** 11-25.

Woodhams, D. C., Kenyon, N., Bell, S. C., Alford, R. A., Chen, S., Billheimer, D., Shyr, Y. & Rollins-Smith, L. A. 2010b. Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata*. *Diversity and Distributions,* 16**,** 703-712.

Woodhams, D. C., Rollins-Smith, L. A., Carey, C., Reinert, L., Tyler, M. J. & Alford, R. A. 2006b. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia,* 146**,** 531-540.

Woodhams, D. C., Voyles, J., Lips, K. R., Carey, C. & Rollins-Smith, L. A. 2006c. Predicted disease susceptibility in a panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases,* 42**,** 207-218.

Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C. J., Rollins-Smith, L. A. & Harris, R. N. 2007b. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation,* 138**,** 390-398.

Yang, L., Weiss, T. M., Lehrer, R. I. & Huang, H. W. 2000. Crystallization of Antimicrobial Pores in Membranes: Magainin and Protegrin. *Biophysical Journal,* 79**,** 2002-2009.

Young, S., Whitehorn, P., Berger, L., Skerratt, L. F., Speare, R., Garland, S. & Webb, R. 2014. Defects in Host Immune Function in Tree Frogs with Chronic Chytridiomycosis. *PLoS ONE,* 9**,** 1.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature,* 415**,** 389.

VITA

Graduate School
Southern Illinois University

David A. Burkart

docodave@gmail.com

North Carolina State University Carbondale
Bachelor of Science, Biological Sciences, December 2007

Special Honors and Awards:

Magna cum laude: North Carolina State University, December 2007

Thesis Paper Title:

Understanding chytridiomycosis resistance by investigating cutaneous defense mechanisms in marsupial frogs

Major Professor: Alessandro Catenazzi

Publications:

Neiles, B., C. S. Carey, A. Araujo, D. Burkart, et al. 2015. Writing your way into high impact factor journals. Bulletin of the Ecological Society of America 96(2): 312-316.

Lynch, R. L., R. Maynard, P. S. Hamilton, and D. Burkart. 2014. Amphibians of the Jama-Coaque Reserve, Manabi, Ecuador.